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Biological methods for detoxification of corn stover and corn starch pyrolysis liquors

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Biological methods for detoxification of corn stover and corn starch pyrolysis liquors

by

Mohammad A. Khiyami

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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For the Major Program

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Abstract

Biological methods were developed to detoxify corn stover and corn starch pyrolysis liquors produced at 400-500°C. Prokaryotic and eukaryotic suspended cells and biofilms were employed for the detoxification process. The continuous of detoxification process was monitored by measuring the change in dissolved oxygen and pH. Total phenolic assay, change in UV absorbance spectra, GC-MS analysis and bioassay were performed to determine the detoxification. *Pseudomonas putida* and *Streptomyces setonii* biofilms, developed on Plastic Composite Supports (PCS) fixed to agitator shaft of benchtop computer controlled bioreactor, detoxified 10 and 25% (v/v) diluted corn stover and corn starch pyrolysis liquor (Dcs and Dst), while mixed culture biofilms detoxified 50% (v/v) Dcs and Dst.

Ligninolytic enzymes of *Phanerochaete chrysosporium* were also employed to detoxify the Dcs and Dst in shake flask cultures. The detoxification was determined by measuring the activity of lignin peroxidase (LiP), manganase peroxidase (MnP), total phenolic compounds reduction, GC-MS analysis, and bioassay. *Ph. chrysosporium* culture detoxified 10 and 25% (v/v) Dcs and Dst, but not 50% (v/v) Dcs and Dst. The involvement of ligninolytic enzymes in the detoxification process were confirmed by adding ligninolytic enzymes inhibitors, sodium azide and cycloheximide to culture medium and by employing concentrated crude enzyme to detoxify 10% (v/v) Dcs.

In a subsequent study, the ligninolytic enzymes were produced by *Ph. chrysosporium* PCS biofilm in stirred tank bioreactor. Fourteen repeated batch fermentations were

performed with different culture conditions. The differences in enzymes production between the batches were determined statistically by comparing the activity of LiP and MnP. All batch conditions evaluated enhanced the production of at least one of the enzymes. In batch C₃V₀ 11 and C₃V₃ 8 (continuous aeration, 300 agitation, and addition of veratryl alcohol on day zero or three) LiP and MnP were produced on day three and reached a peak on day six. However, in batch C₃VM₀ 14 (continuous aeration, 300 agitation, and addition of veratryl alcohol and MnSO₄ on day zero) LiP and MnP were produced earlier, on day three, and decreased by day six.

General Introduction

Introduction

Lignocellulosic materials are huge renewable resource for the production of desirable chemicals such as organic acids. Lignocellulosic material, consisted of lignin, hemicelluloses and cellulose, to be used as carbon and energy sources in fermentation industries have to be available in a simple and safe way, at acceptable prices, and in a way not affecting the environment. Levoglucosan (1,6-anhydro- β -D-glucopyranose), the main component of the highly viscous liquid generated during the pyrolysis process of biomass, is attractive due to the low cost and the possibility of bioconversion by microorganisms directly or indirectly as a potential feedstock for the fermentation industries (16). Unfortunately, other compounds include aromatic species, aldehydes, furan and furfuryl derivatives are found in the pyrolysis liquid. When considering this material as a fermentable substrate, these components are of concern, because they contain compounds that are toxic or inhibit microbial growth (109). However, some microorganisms have the ability to grow and detoxify some of the previously mention inhibitor compounds.

Microorganisms that utilize organo-pollutants are ubiquitous in nature and for both economic and ecological reasons the biological degradation has become a desirable method for the treatment of organo-pollutants. The majority of biological methods and processes for the remediation of organo- pollutants to date have concentrated upon prokaryotes such as organic-solvent-tolerant bacteria, with comparatively limited studies on eukaryotic in particular white-rot fungi (104). As a variety of pollutants has been shown to be biodegradable by organic-tolerant Gram negative and positive bacteria as single species and

mixed cultures under laboratory conditions, the biological treatments technology seem to be a promising tool. The biodegradation of a mixture of pollutants compounds can occur when these components are in low concentrations, water soluble and degrade by same microbial enzymes. Although there are some microorganisms, which can assimilate organic compounds, they do so only when the solvents concentration is very low (120). The microbial biofilms which is the natural form of cell immobilization are more resistance to high concentration of organic compounds and increase the biodegradation rate (25, 62, 63). To develop and enhance the biodegradation process of pollutants in the presence of mixture of these pollutants proper microbes that are tolerant to these pollutants would be essential.

The goal of this study is to develop biological protocol for corn stover and corn starch pyrolysis liquors bioremediation (detoxification) and free the levoglucosan for the microbial fermentation. The first paper demonstrates the effectiveness of *Pseudomonas putida* and *Streptomyces setonii* suspended cell and PCS biofilms reactor to detoxify different concentrations of corn stover and corn starch pyrolysis liquors. The second paper demonstrates the effect of ligninases of *Phanerochaete chrysosporium* to detoxify different concentrations of corn stover and corn starch pyrolysis liquors. The third paper demonstrates the effect of *Ph. chrysosporium* PCS biofilm stirred tank with different culture conditions to increase the ligninases production.

Dissertation organization

This dissertation follows an alternative format and is divided into three papers. Each paper contains an abstract, introduction, materials and methods, results, discussion, and references with tables and figures included in the text. The papers are written to conform to

the specifications of Applied and Environmental Microbiology, the journal to which the papers will be submitted. A general introduction chapter including a literature review and general conclusion chapter has been included. All experiments, data collection, and data analysis were performed by the candidate.

Literature review

Pyrolysis

Pyrolysis is defined as a chemical degradation reaction caused by thermal energy alone. Chemical degradation refers to the decomposition of biomass that occurs during pyrolysis with the formation of molecules smaller than the starting material (90).

The pyrolysis process

The pyrolysis process can be carried out at different high temperatures (250-800°C) in which the biomass is rapidly heated in the absence of oxygen (16, 44). As the biomass is heated, a sequence of physical and chemical changes takes place to generate a mixture of solid char, condensable liquids, and gases. After cooling and condensation, a dark brown mobile material termed pyrolysis-oil or pyrolysis liquid is formed. The process aim is to optimize the formation of condensable organic vapors, with a minimum of gas and char is known as fast pyrolysis. Based on dry feed of the biomass and reactor conditions, fast pyrolysis yields between 40 and 65% (wt/wt) organic condensate, 10 to 20% char, 10 to 30% gases and 5 to 15% water. These ratios depend upon the feedstock, time and temperature of the pyrolysis reactor (44).

The pyrolysis liquid characteristics

The pyrolysis oil typically is a dark brown free flowing liquid (44). Depending upon the initial feedstock and the mode of fast pyrolysis, the color can be almost black through dark red-brown to dark green, being influenced by the chemical composition. The liquid has a distinctive odor, an acrid smoky smell, which can irritate the eyes if exposed for a prolonged period. A wide range of viscosities have been reported for pyrolysis oil which reflect differences in average molecular weight or extent of cracking of oligomers and monomer fragments, the relative success in recovery of the volatile organics is from the carrier and pyrolysis gases, the water content, and possibly the content of char fines (103, 44).

The economics of the fast pyrolysis process

Pyrolysis oil can be produced for 0.13 to 0.16 US\$ per liter of wet oil (\$6.50 to \$7.0/GJ) with feedstock is 44 to 60 US\$ per dry ton. A zero cost for feedstock, as in the case for waste biomass, the predicted production cost is \$2.0/GJ. The economics for pyrolysis oil production are encouraging for the higher valued applications, such as fuel oil in any static heating of electricity generation application. The pyrolysis oil also has a range of specialty such as levoglucosan and commodity chemicals such as resins and fertilizers, which was the target for several research groups. The levoglucosan from pyrolysis oils has been used as a substrate for ethanol fermentation (44). From the plant biomass the specialty and commodity chemicals include organic acid and phenolic compounds, which can be used for phenol-formaldehyde resins (44). Unfortunately, phenolic compounds and some commodity chemicals are toxic to microbial growth and limited the utilization of levoglucosan, which is a target substrate in the fermentation industry (44).

Levoglucosan

Levoglucosan (1, 6-anhydro- β -D-glucopyranose), is dehydrated glucose and contains an intermolecular glucoside bound. It is the main product of pyrolysates of cellulose or starch (144). Several studies mentioned the potential of utilizing levoglucosan as precursor for the synthesis of rare sugars such as 1,6:3,4-dianhydro- β -D-talopyranose, herbicidal derivatives and some antibiotics such as indanomycin (47, 87,148). Also, levoglucosan can be fermented to ethanol and butanol (100). Microbial fermentation of levoglucosan can occur at indirectly by hydrolyzing levoglucosan with mild acid to produce glucose or directly by eukaryotic or prokaryotic microorganisms (92, 93, 109, 152). Eukaryotic microorganisms, which have levoglucosan kinase, convert levoglucosan to glucose-6-phosphate. A variety of fungi and yeast were screened for their ability to utilize and ferment the levoglucosan produced from lignocellulosic pyrolysis, and some of them produced high ethanol yields (109). *Aspergillus terreus* converts levoglucosan generated from cellulose into itaconic acid in the same yield and at the same fermentation rates as glucose (92). *Aspergillus niger* fermented levoglucosan generated from starch to citric acid at a yield of 87.5% (152). Prokaryotic microorganisms metabolize levoglucosan through at least 3 steps and require NAD^+ as a cofactor (150). A novel enzyme called levoglucosan dehydrogenase isolated from *Arthrobacter* sp catalyzes the dehydrogenation of levoglucosan to 3-keto levoglucosan, then to 3-keto glucose, and finally to D-glucose by using NAD^+ as an electron acceptor (93) (Figure 1).

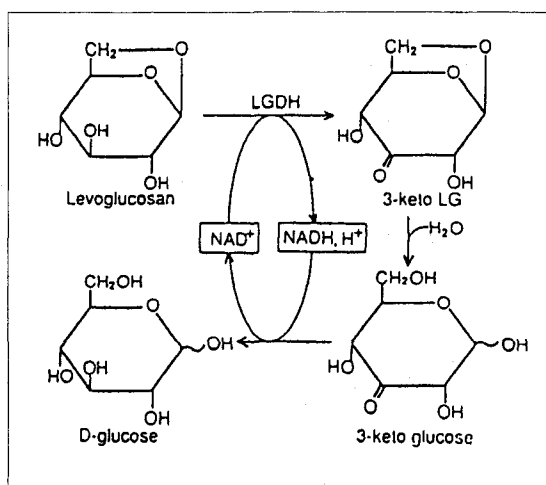


Figure 1. A possible reaction scheme of the conversion of levoglucosan to glucose by levoglucosan dehydrogenase (LGDH) identified in *Arthrobacter* sp (93).

Corn stover pyrolysis liquid

Corn stover is lignocellulosic feedstock after harvesting of corn. It contains 38% cellulose, 32% hemicelluloses and 20% lignin (17). In the fast pyrolysis process, cellulose is pyrolyzed predominately to the monomer levoglucosan with yields ranging from 38 to 58% of the initial cellulose content (109). In the presence of alkali catalysts, which are naturally found in biomass material, carbohydrate rings are split open with the production of hydroxyacetaldehyde, organic acids, furfurals, and other oxygenated compounds (44). However, the existence of these compounds and their chemical compositions can be changed based on the pyrolysis temperatures (90). For instance, the analysis of cellulose pyrolyzed at 850°C by gas chromatography/mass spectroscopy (GC-MS) showed numerous compounds such as furanmethanol, 1,3-dioxolane-4,5-diol, 1,3-dihydroxybenzene, 1,2-dihydroxyethane, hydroxy acetic acid, diethyleneglycol, glycerol and levoglucosan. However, when cellulose is pyrolyzed at 450°C fewer compounds were obtained such as 1,2-dihydroxyethane,

hydroxy acetic acid, diethyleneglycol, glycerol and levoglucosan (90). Also, under faster heating conditions and higher temperatures pyrolysis of hemicelluloses gives rise to analogous families of chemical compounds such as levoglucosen, hydroxyacetaldehyde, organic acids, furfurals, and other oxygenated compounds (44). Lignin in the presence of alkali catalysts pyrolyze to monocyclic and noncondensed bicyclic aromatic material with a high phenolic content. These compounds (Table 1) are mainly derived from the depolymerization of different lignin sources (hard wood, soft wood, and grass) at about 400-450°C (44). These phenols have zero, one or two methoxy groups, depending upon the plant species pyrolyzed (44).

Table 1. Phenolic compounds derived from the depolymerization of hard and soft wood lignin at 400-450^a.

Compound	Compound
Phenol	Vanillin
<i>o</i> -Cresol	Isoeugenol
<i>m</i> -Cresol	Ethylhydroxymethoxybenzaldehyde
<i>p</i> -Cresol	1,2,3,-Trihydroxybenzene
Guaiacol	Allylsyringol
Propenylphenol	Propylsyringol
2-Methoxy-4-methylphenol	Syringaldehyde
Catechol	Dimethoxyhydroxybenzaldehyde
2,3-Dimethoxyphenol	1,2,4-trihydroxybenzene
Hydroquinone	Propenylsyringol
Methoxybenzenediol	Methylsyringo
Syringol	Dimethylbenzenedio
Dimethoxyphenol	Dimethoxymethylphenol
Methylbenzenediol	Methoxymethylphenol
Eugenol	Methoxyhydroxybenzaldehyde

^a Some of the phenolic compounds list such as catechol, eugenol, and vanillin have a relatively high commercial value (44).

Starch pyrolysis liquid

In industry, starch is subjected to a variety of chemical modifications to improve the functionality for specific applications (90). For example, the preparation of starch succinate or alkenylsuccinates by reaction with succinic anhydride or its derivatives. Another example is the cross-linking of starch, which is done by reacting starch with small amounts of reagents with bifunctional group such as epichlorohydrin, acid anhydrides, etc. Such modified starch has numerous applications in food and pharmaceuticals.

Little information is available about modified starch pyrolysates. Some analytical studies (90) identified different compounds such as 2-furaldehyde, 2-furanmethanol, 2(5H)-furanone, 5-methyl-2-(3H)-furanone, 5-methylfurfural, phenol, 3-methyl-5-methyliden-2(5H)-furanone, 5-hydroxymethylfurfural, 3-methoxy-5-methyl-2(5H)-furanone, 1,6-anhydro- β -D-glucopyranose, and 1,6-anhydro- β -D-glucofuranose in the pyrolysates of starch cross-linked with epichlorohydrin (SE), starch cross-linked with epichlorohydrin in the presence of ammonia (SEA), and starch cross-linked with epichlorohydrin in the presence of ammonia and 3-chloro-2-hydroxypropyltrimethyl-ammonium chloride (SQEA). The pyrolysis was performed at 600°C and the identified compounds were separated by GC-MS on DB-5ms capillary column 30 m long, 0.25 mm id, 0.25 μ m film thickness. However, at the lower pyrolysis temperature pyrolysate composition becomes less complex (90).

Toxicity of the pyrolysis liquid

Toxicity of pyrolysis liquid results from aromatic compounds generated during the pyrolysis process. In low temperature (400 to 450°C), pyrolysis oil (pyrolysis liquid) does not contain polycyclic aromatic and appears not to be carcinogenic (44). However, under high

temperatures the condensable organic yield is relatively low and contains benzene and polycyclic aromatic compounds, which are more carcinogenic than benzo(a)pyrene (44). More recent study has shown that whole fast pyrolysis oils dose contain mutagens based on the Ames Test (44).

Lignocellulosic pyrolysis liquid showed numerous toxic compounds with levoglucosan, such as benzene, toluene, and ethylbenzene. These compounds are important as industrial solvents and intermediates in chemical synthetic processes, but they are toxic to many microorganisms. The toxicity of the organic solvents is expressed in terms of the partitioning of the solvent between octanol and water ($\log P_{ow}$). The organic solvents with a $\log P_{ow}$ 1 to 5 are extremely toxic for microorganisms and other living cells, because they partition preferentially in cytoplasmic membranes disrupting membrane integrity and function membrane (113). The hydrophobicity and toxicity of organic solvents lead to the classification of these compounds in five different groups: (a) short-chain alcohols such as methanol, ethanol, and 1-butanol, (b) weak acids such as acetic acid, and butyric acid (c) lipophilic solvents such as hexane, decane, and Hexadecane (d) aromatic solvents such as benzene, toluene, styrene, and tetralin (e) aromatic alcohols such as phenol, 4-chlorophenol, 2,4-dichlorophenol, 2,4,5-trichlorophenol, and pentachlorophenol (69). The last two groups are unsaturated compounds characterized by having at least one six carbon ring and possess uniform π -orbitals which result in increased stability of the compounds. In pyrolysis liquids the aromatic compounds beside the aldehydes, furan, and furfuryl derivatives are considered toxic or inhibitors of microbial growth (109). The postpyrolysis clean-up procedures are necessary for enhancing microbial fermentation of levoglucosan (92, 109).

Detoxification (clean-up) methods for pyrolysis liquids

Conventional methods

Solvents and activated carbon are the most common methods used to clean-up the pyrolysis liquids before the fermentation process (92, 109,152). The extraction with activated carbon required mixing the supernatant of the diluted pyrolysis liquid and the activated carbon. The mixture warmed slightly and gently stirred for 2 hr. This process needs 300 g of activated carbon to extract toxic compounds from one liter of pretreated lignocellulosics diluted pyrolysis to produce fermentable levoglucosan (109).

Biological method

To date, no biological clean-up method was employed with the pyrolysis liquids. However, biodegradation and bioremediation are employed with many reluctant chemical, such as herbicides, insecticides and organic solvents. Organic solvents dissolve in microbial cell membranes, disturbing its integrity and affecting permeabilization. Thus, results in impairing vital cellular functions, such as loss of ions, metabolites, lipid, and proteins, the dissipation of the pH gradient, electrical potential and the inhibition of membrane protein functions (113), which can lead to cell death and lysis (39,111). These toxicity effects are similar for a variety of microorganisms, whether Gram-positive and Gram-negative bacteria, as well as yeast, which are killed by high concentration of these compounds (69). However, some microorganisms such as *Pseudomonas* sp. and *Streptomyces* are able to metabolize some of those compounds (32, 112,147). Thus, these microorganisms can be employed in a biological detoxification treatment.

Mechanisms of resistance to toxic compounds

Bacteria possess several possible mechanisms leading to solvent-tolerance. However, not all known or proposed organic solvent-tolerance mechanisms in bacteria may operate in every microorganism and this variability of mechanisms results in a variety of detoxification mechanisms. Sardesai and Bhosle (120) reviewed the proposed solvent-tolerant mechanisms in Gram-positive bacteria such as *Bacillus*, *Rhodococcus*, and *Arthrobacter*, which include “(a) protection rendered by endospores, (b) induction of general stress regulon, leading to the production of general stress proteins, (c) solvent deactivating or emulsifying enzymes”.

Ramos et al. (113) reported a numbers of elements involved in the response to toxic chemical in Gram-negative “(a) rigidification of the cell membrane via alteration of the phospholipids composition, (b) alterations in cell surface that make the cells less permeable, (c) efflux of toxic compounds in an energy-dependent process, and (d) formation of vesicles that remove solvent from the cell surface (e) metabolism of toxic hydrocarbon, which can contribute to their transformation into nontoxic compounds”.

All naturally occurring organic compounds in the biosphere can be degraded by microorganisms (3). The term biodegradation refers to the ability of microorganisms to mineralize or convert organic molecules to different forms (3). Heterotrophic microorganisms utilize organic compounds as both carbon and energy sources for growth and maintenance of the cell. This is accomplished by the sequential action of enzymes to convert a substrate to a form that can be utilized in the central metabolic pathways (51, 94). The term mineralization refers to the conversion of an organic substrate to CO₂ and H₂O. Generally, a part of the mineralized molecule is incorporated into cell material. However, the white-rot fungi are so far the only organisms know that degrade lignin or similar aromatic compounds

to CO₂ and H₂O but they cannot use these substrates as sole carbon and energy source (20, 51, 104).

Aromatic compounds degradation

The first demonstration of the microbial ability to utilize aromatic compounds was reported in 1908. Although both mono and poly-aromatic compounds can be oxidized by microorganisms (bacteria and fungi), this section focuses on aerobic bacterial catabolic pathways that metabolize single aromatic compounds which are dominant in corn stover and modified corn starch pyrolysis liquors such as SE, SEA, and SQEA. Also, this section focuses on oxidation of aromatic compounds by the white-rot fungi ligninases. Studies have shown that aromatic hydrocarbons are metabolized a variety of ways, often to one or few common ring-fission substrates (51).

Biodegradation of an aromatic molecule involves two steps: activation of the ring, and ring cleavage (Figure 2). Activation involves the incorporation of molecular oxygen into the ring, that is, dihydroxylation of the aromatic nucleus (51). This step is achieved by enzymes known as oxygenases. Monooxygenases, characteristic of fungi and other eukaryotes, catalyze the incorporation of a single atom of oxygen to form an epoxide, which can then undergo hydration to yield *trans*-dihydrodiols (21). Dioxygenases, characteristic of bacteria, catalyze the incorporation of two atoms of molecular oxygen at one time to form a dihydrodiol. These dioxygenase reactions have been shown to occur for aromatic compounds such as phenol, benzene, toluene, xylenes and naphthalene (10, 34, 52, 54).

Dihydrodiols are oxidized to dihydroxylated derivatives such as catechol (1,2-dihydroxybenzene) and protocatechuate (3,4-dihydroxybenzoate), which are precursors to ring

cleavage. Catechol or protocatechuate can be oxidized either via the *ortho*-pathway which involves cleavage of the bond between carbon atoms of the two hydroxyl groups to yield muconic acid (Figure 3), or via the *meta*-pathway which involves cleavage of the bond between a carbon atom with a hydroxyl group and the adjacent carbon atom to yield 2-hydroxymuconic semialdehyde (Figure 4), these compounds are then degraded to form acids which are readily utilized by microorganisms for cell synthesis and energy (51).

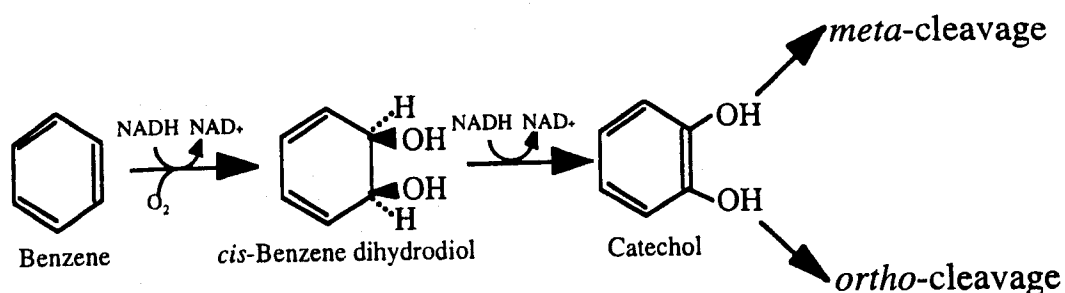


Figure 2. Pathway of benzene degradation by bacteria.

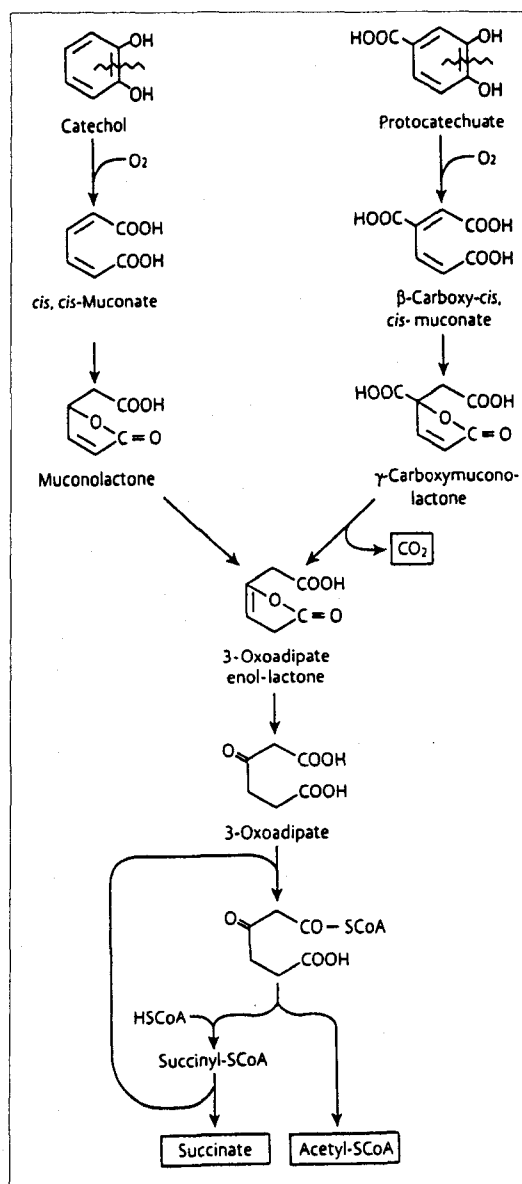


Figure 3. Dissimilation of catechol and protocatechuate by the involving *ortho*-cleavage (51).

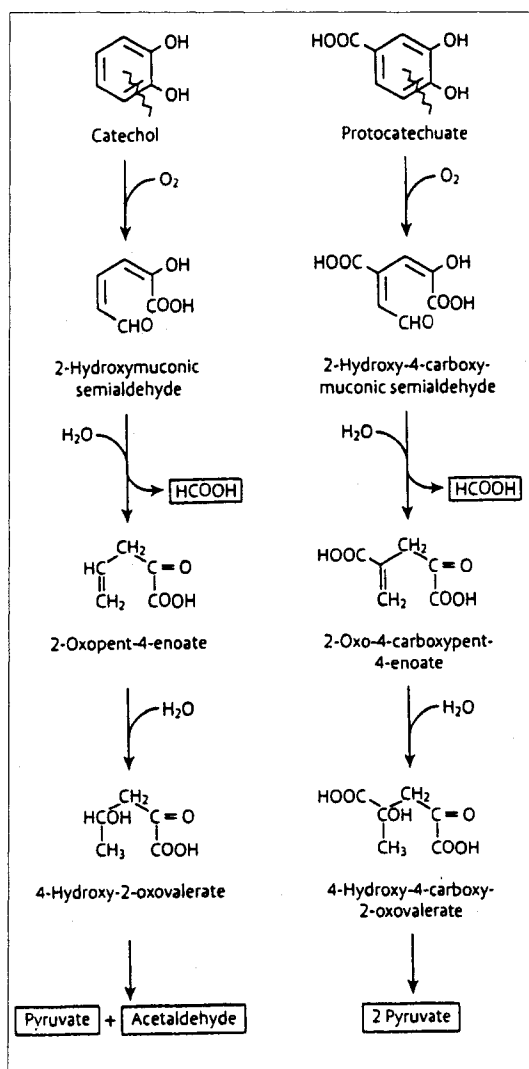


Figure 4. Dissimilation of catechol and protocatechuate by the involving *meta*-cleavage (51).

The ability of white-rot fungi to degrade lignin and a wide variety of structurally diverse aromatic compounds is dependent upon the lignin-biodegrading system of these fungi (1, 38, 102) (Figure 5). The fungal peroxidases, lignin peroxidase (LiP), manganese peroxidase (MnP) and veratryl alcohol are secreted into the extracellular medium in response to nutrient limitation. Veratryl alcohol acts as an electron transfer mediator between LiP and

lignin, or may just protect LiP from inactivation. Hydrogen peroxide, produced from the action of glyoxal oxidase (also secreted by the fungi) on glyoxal and O_2 , oxidizes the lignin peroxidases, which then act upon the lignin molecule, resulting in the formation of lignin cation radicals. These lignin radicals then degrade non-enzymatically to aromatic and aliphatic products, which are mineralized intracellularly to CO_2 . Also, the lignin radicals generated can carry out a variety of reactions, including benzylic alcohol oxidation, carbon-carbon bond cleavage, hydroxylation, phenol dimerization/polymerization, and demethylation (104).

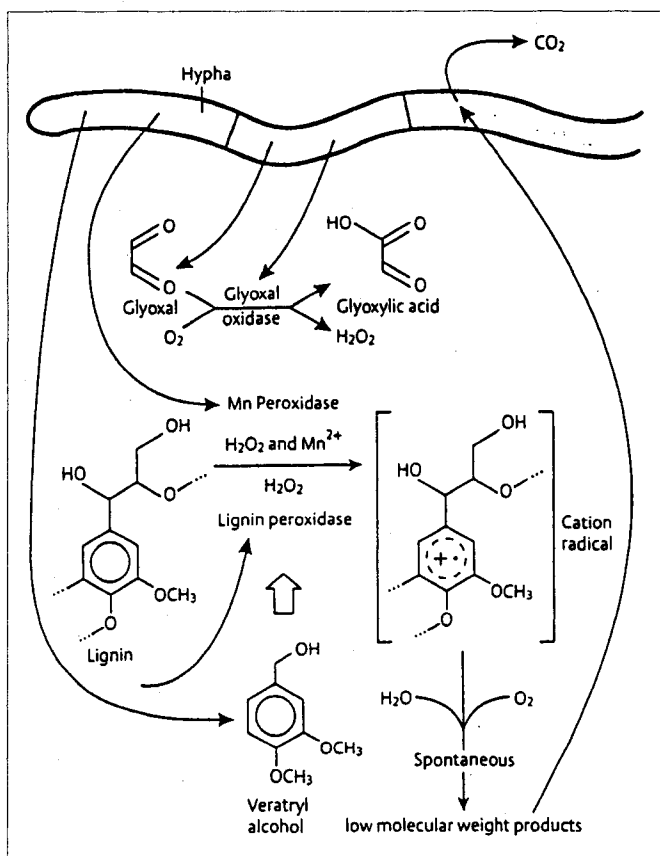


Figure 5. Overview of the ligninolytic system in *Phanerochaete chrysosporium* (51).

The whit-rot fungi enzymology has been the subject of considerable attention since their discovery in 1983 (59, 134). Lignin peroxidases are highly potent oxidizing agents and their catalytic mechanism is similar to that of other peroxidase. The lignin peroxidase (LiP) enzyme is thought to be oxidized by a two-electron transfer from H_2O_2 to form Fe^{IV} cation radical called compound I (Figure 6), which oxidizes a substrate molecule (e.g. veratryl alcohol) by one electron, forming a free-radical product and reduced by one electron to form compound II, which oxidizes another substrate molecule by one electron forming second free-radical, with the enzyme being returned to the resting state in the process (20, 68).

Manganese peroxidase (MnP) is typically explained by the ability of the enzyme compound I and II to oxidize Mn^{2+} to Mn^{3+} (145), which can not be replaced by other metals (58). The Mn^{2+} oxidation rate is increased by presence of organic acid, such as oxalic and lactic acid (57). Oxalate, glyoxylate and malonate are produced *de novo* by white-rot fungi. The organic acids are able to chelate Mn^{3+} and form stable complex (Figure 6), which can oxidize various phenolic compounds through phenoxy radical formation (101).

The biological degradation of such pollutants occurs in many cases as part of a co-metabolic activity, where the organism's growth is maintained by specific substrates and the detoxification activity of other materials that are nongrowth substrates ensues (15, 55). Many bacteria and fungi can accomplish simple transformations of organic substrates but often fail to complete the conversion of a toxicant to CO_2 or they generate toxic intermediates that can impair microbial growth. However, many pure and mixed culture microorganisms can detoxify monocyclic compounds have been isolated and studied (2, 5, 22, 25, 53, 60, 74, 85, 128,143). The use of microorganisms either naturally occurring or introduced to the contamination area to detoxify pollutants is called bioremediation (3).

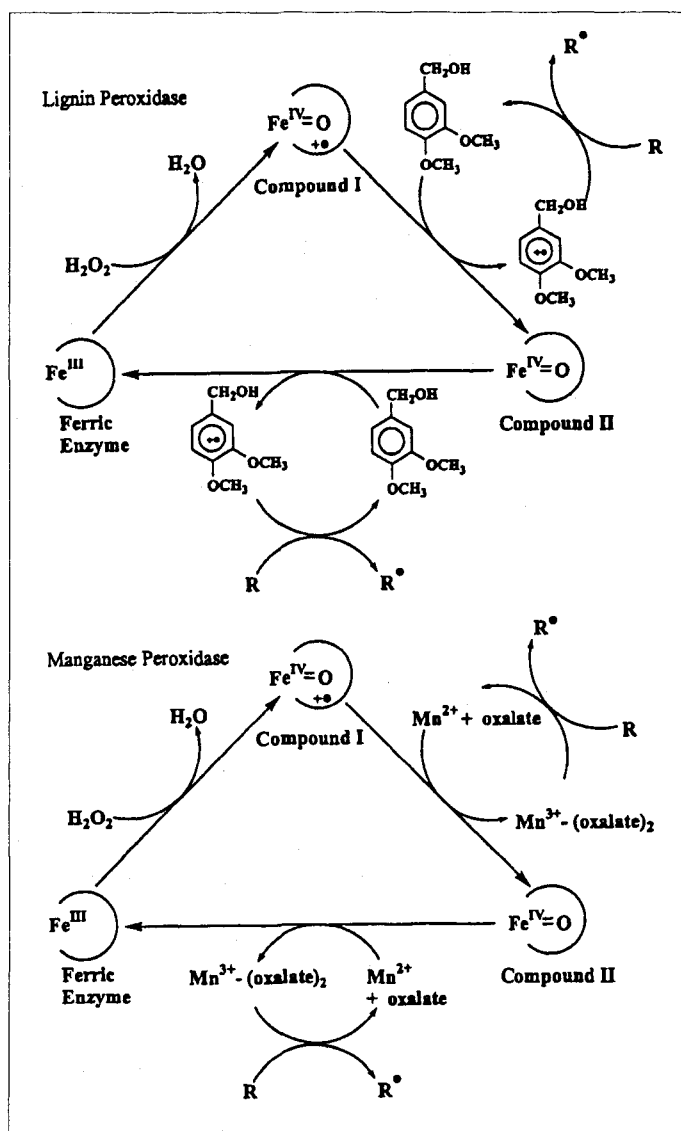


Figure 6. Catalysis and mediation by lignin peroxidase and manganese peroxidase (20).

Bioremediation

The goal of bioremediation is to degrade pollutants to concentrations that are either undetectable or to concentrations below toxicity limits. Bioremediation is the technology of choice for eliminating organic pollutants because it is safe and less expensive (3). Most bioremediation currently conducted on a commercial scale utilizes prokaryotes (104).

However, eukaryotes such as white-rot fungi offer advantage in the diversity of compounds they are able to oxidize (87). Bioremediation can be separated into two approaches *in situ* treatments in which contaminated material is not removed from site (3). *In situ* bioremediation has the advantage of relatively low cost but the disadvantage of being less subject to rigorous control (3). The second approaches for the bioremediation is bioreactors. Bioreactors have been developed for the treatment of liquid, solid, or gases (3). Such bioreactors are often used for the destruction of residual chemicals, by-products, and other waste from manufacturing facilities, and they are attractive because of the frequently low cost compared to other means of waste disposal (3).

Two types of bioreactors have dominated laboratory studies to control culture environment and bioremediate pollutants. These bioreactors are suspended culture or immobilized cells (3). In the first type, microorganisms are suspended continuously and they grow freely in a submerged liquid or are attached to the contaminated material that is maintained in suspension. In the second type, immobilized cells reactors, microorganisms are entrapped or fixed on some type of matrix, so they are not removed as the effluent leaves the reactor, except if desired. Bioreactors can be operated as continuous system, in which the contaminated liquid or suspension enters and treated liquid is removed continuously or they can be operated as batch system. Batch operations are most common for the treatment of soil, sludge, or other solids and for biodegradations that requires long periods of time (3). Bioremediation bioreactors are inoculated with a single species or a mixture of microorganisms able to function effectively under the controlled conditions and some of these microorganisms develop biofilms, a natural form of cell immobilization.

Bioremediation by suspended cell and biofilm cultures

Pseudomonas putida, *Streptomyce setonii*, and *Phanerochaete chrysosporium* as pure and mixed culture have been used as suspended cell, biofilm or immobilized cell bioreactors for biodegradation of numerous compounds (2, 3, 5, 22, 25, 53, 60, 69, 74, 85, 128, 143). The biofilm cultures show greater resistance to many substances such as antibiotics, chlorine, phenols and detergents (27). Also, biofilm reactors demonstrate enhanced rates of toxic compounds biodegradation compared to suspended cell cultures (63, 83, 117, 125, 142).

Pseudomonas putida

P. putida has the ability to develop biofilm and degrades numerous aromatic hydrocarbons as sole carbon and energy sources (Table 2) (11, 13, 83, 142, 151). Moreover, the *P. putida* immobilized culture showed better performance in degradation than the suspended-culture. *P. putida* in fluidized-bed bioreactors where cells were immobilized within calcium alginate gel beads demonstrated increase phenol degradation ≤ 4 g/day (57). In addition, several studies observed that the ability of *P. putida* to degrade various aromatic compounds was more sufficient in a mixed culture rather than in a pure culture (5, 13, 123, 124, 136).

Streptomyce setonii

The filamentous, soil-living, Gram-positive bacteria, *Streptomyces* can degrade lignin derived aromatic compounds and naturally develop biofilms (7, 24, 30, 64, 67, 105, 106, 130, 131). *Streptomyces viridosporus* was reported to degrade corn stover lignocellulose successfully and produce peroxidase, xylanases, esterases and endoglucanases (31).

Streptomyces cyaneus degraded lignocellulose during growth in solid-substrate fermentation on wheat straw, and it produces extracellular phenol oxidase (12). For bioremediation of a mixture of aromatic compounds the pure culture approach has serious limitation (9, 88, 114, 115). In other words, the degradability of one component by a pure culture was strongly affected by the presence of the other compounds. Normally, the microbial degradation of an aromatic compound mixture can be strongly impacted by other substituents in the mixture (82, 118). Whereas, bacterial mixed culture with varied physiology was shown to have a higher biodegradation rate than individual pure cultures (50).

Table 2. Compounds degraded by *pseudomonas putida*.

Compound	Culture	Reference
Chlorobenzene	Suspended cell	81
4-thylbenzene	Suspended cell	110
Toluene, Benzene, Phenol	Suspended cell	114
<i>p</i> -Cresol	Suspended cell	151
Methylnaphtalene	Suspended cell	11
4-nitrophenol	Suspended cell	96
Phenol	Suspended cell	91
Phenol	Suspended cell & immobilized cell	61
Phenol	Suspended cell & immobilized cell	63
Phenolics compounds	Immobilized cell	62
Toluene and ethanol	Biofilm	142
Toluene	Biofilm	149
Naphthalene	Suspended cell & biofilm	125
Low molecular weight aromatic compounds	Mixed culture biofilm	13
Benzene, Toluene, Ethylbenzene, Xylene	Immobilized mixed culture	124
Polycyclic aromatic hydrocarbon	Mixed culture	136
Toluene, Benzene, Xylene	Mixed culture	5

Mixed culture

Mixed-culture microbial communities are designed to degrade a wide range of substituted aromatic compounds as sole carbon source in aerobic batch cultures (48). The mixed cultures are considered to have several advantages over pure cultures, such as greater stability and increased metabolic capabilities. These characteristics enable the mixed culture to overcome limitations for the complete biodegradation of toxic compounds (37, 48). Immobilized mixed culture accelerates biodegradation while, enabling cell mass reuse (12). For example, a ten member microbial consortium consisting of eight phenol-degrading and two non-phenol-degrading strains of bacteria was developed and maintained in a fed batch reactor by feeding 500 mg /l phenol for four years (6). The mixed culture degraded 99% of 500 mg/l phenol after 24 hours incubations. Characterization of mixed culture members revealed that it consisted of 4 principal genera, *Bacillus*, *Pseudomonas*, *Rhodococcus* and *Streptomyces* (6).

Phanerochaete chrysosporium

Wood rotting basidiomycetes have been categorized into two groups, white-rot and brown-rot fungi, according to their ability to remove lignin from wood components during decay. The white-rot fungi are capable of degrading lignin to CO₂ and H₂O₂ by a ligninase degrading system. Bioremediation technologies using white-rot fungi have been widely studied with crude oil polluted soil and a variety of persistent environmental pollutants (75, 104). White-rot degradation system, in particular *Ph. chrysosporium* consists of peroxidases, laccase, H₂O₂-producing enzymes, veratryl alcohol, manganese and oxalate, cellobiose

dehydrogenase, a trans-membrane methyl transferase, and trans-membrane redox potential (20).

For ligninolysis to occur an alternative carbon source is needed since lignin degradation product are not utilized as a carbon and energy source (80). Lignin degradation is a secondary metabolism, triggered by limiting cultures nutrients (i.e. nitrogen, carbon or sulfur) (78, 79). Under laboratory conditions, ligninolysis is usually induced by low levels of nitrogen rather than limiting carbon or sulfur, since ligninolysis is not as pronounced under these conditions (78). Most biodegradation studies using *Ph. chrysosporium* have been performed with the organism in flask culture (8, 19, 46, 119). These studies suggested that enzymes involved in ligninase degradation were also involved in xenobiotic degradation.

The potential applications of *Ph. chrysosporium* and their enzymes are gaining increasing importance in the bioremediation of industrial waste and of a vast range of xenobiotics environmental pollutants (104). Many studies reported the ability of white-rot fungi to degrade organic pollutants, such as munitions waste (73), pesticides (4), polychlorinated biphenyls (97), polycyclic aromatic hydrocarbons (43), bleach plant effluent (45, 86, 99), synthetic dyes (56), synthetic polymers (94), and wood preservatives (116). Although the exact mechanisms of xenobiotics biodegradation by white-rot fungi are not known in many cases, there is considerable evidence to suggest that ligninolytic enzymes are responsible.

Since, it is known that *Ph. chrysosporium* has remarkable abilities to degrade a wide variety of chlorinated and nonchlorinated environmental pollutants, as well as many other compounds (Table 3), various methods of culturing white-rot fungi have been investigated in order to maximize the production of lignin-degrading enzymes. Attempts to scale up to larger

flasks or to shallow trays resulted in lower activity (76, 100). Agitated submerged cultures in Erlenmeyer flasks or in stirred tank fermentors permitted good growth as mycelial pellets, but only traces or no ligninase activity (80, 141). Immobilization of *Ph. chrysosporium* mycelia cell culture was more effective in promoting cell growth and increasing the peroxidases production compared to conventional stationary phase liquid culture (126). Enzyme production by *Ph. chrysosporium* was maximized in fixed-bed tubular bioreactor, filed with cubes of nylon sponge, to reach 1293 U/l of MnP and 225 U/l of LiP (89). Also, the ligninases production was scaled up in a fixed trickle bed reactor by controlling oxygen transfer efficiency and *Ph. chrysosporium* biofilm thickness (14, 70).

The biofilm phenomenon can be advantageous in bioremediation. Several bacteria and fungi have been used as biofilm or in immobilized cells system and a number of compounds are readily biodegraded by these system including benzene, toluene, ethylbenzene, naphthalene, phenanthrene, pyrene, anthracene, benzoanthracene, chrysene, and benzopyrene (3, 138). Microbial biofilm are often sufficiently thick and extensive to be visible to the unaided eye, and they may contain millions of cells in arrangements that facilitate the stable microbial mat.

Table 3. Organic compounds degraded by *Ph. chrysosporium*.

Polycyclic Aromatic Compounds	Chlorinated Aromatic Compounds
Benzo[a]pyrene	4-Chlorobenzoic Acid
Biphenyl	Dichlorobenzoic Acid
2-Methylnaphthalene	2,4,6-Trichlorobenzoic Acid
Phenanthrene	4,5-Dichloroguaiacol
Benz [a] anthracene	6-Chlorovanillin
Pyrene	4,5,6-Trichloroguaiacol
Anthracene	Tetrachloroguaiacol
Perylene	Pentachlorophenol
Dibnzo[p]dioxin	2,4,5-trichlorophenol
Chrysene	2,4,6-trichlorophenol
Naphehalene	2,4-Dichlorophenol
Polycyclic Chlorinated Aromatic Compounds	3-Chloroaniline
DDT [1,1 ,1-Trichloro-2,2-bis(4-chlorophenyl)ethane]	2,4-Dichloroaniline
Chlordane [Octachlorohexahydromethanoindane]	2,4,5-Trichlorophenoxyacetic Acid
Lindane [1,2,3,4,5,6-hexachlorohexane]	Chlorinated Alkyhalides
Toxaphene [Mixture of chlorinated camphenes]	Lindane
2,3,7,8-Tetrachlorodibenzo-p-dioxin	Chlordane
3,4,3',4'-Tetrachlorobiphenyl	Biopolymers
2,4,5,2',4',5'-Hexachlorobiphenyl	Lignin
Aroclor 1254	Cellulose
Aroclor 1242	Kraft lignin
2-Chlorodibenzo[p]dioxin	3-Chloroaniline-lignin conjugate
Dicofol [2,2,2-Trichloro-1,1-bis(4-chlorophenyl) ethanol]	3,4-Dichloroaniline-lignin conjugate
Munitions	Dyes
TNT [2,4,6-Trinitrotoluene]	Crystal violet
DNT [2,4-Dinitrotoluene]	Pararosaniline
HMX [Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine] Nitroglycerin	Cresol red
RDX [Hexahydro-1,3,5-trinitro-1,3,5-triazine]	Bromphenol blue
	Ethyl violet
	Malachite green
	Brilliant green

The above is a partial list of compounds degraded by *Ph. chrysosporium* compiled from references [18, 49, 65, 66, 104].

Biofilm formation

Biofilms are a natural form of cell immobilization (84), in which microbial cells are attached to a solid surface (a substratum). Biofilm are made up of microbial cells and

exopolysaccharide (EPS) (23) and the vast majority of bacterial EPS are made up of polysaccharide (26).

Biofilm formation can occur by at least three mechanisms (129). First, the redistribution of immobilizes cells by surface motility (33). Second the binary division of immobilization cells (71). While the cells divide the new cell spread outward and upward from the attachment surface to form cell clusters similar to the colony formation on agar plates. Third, is the attachment of cells from the bulk fluid to the developing biofilm (135). The relative contribution of each of these mechanisms depends on the organisms involved, and the physical and chemical conditions of the environment (129).

Biofilm formation normally develops in five-stage process (Figure 7). Stage one, the planktonic cell approaches the surface (a substratum) so closely that its motility is slowed (35, 129, 146). Stage two, the cells attach to the surface or other cells previously attached to the surface and exhibit reversible and irreversible adsorption processes. The reversible association consists of long range interaction forces, such as van der Waals forces, electrostatic forces and hydrophobic interactions (77). In this stage, the attach cells are surrounded only by small amounts of EPS material and many are capable of independent movement by pilus-mediated twitching or gliding (98). These cells may leave the surface to resume the planktonic lifestyle (129). Stage three, is the early development of biofilm architecture. The attachment cells exude EPS and develop basic microcolony and water channel architecture beside alter their physiological processes in response to conditions, such as growing anaerobically (129). Stage four, the maturation, resulted in the generating complex architecture, channels, pores, and redistribution of cells away from the surface (36). Stage five, the detachment, resulted in individual microcolonies that may detach from the

surface or may give rise to planktonic revertants that swim or float away from these architecture, leaving hollow remnants of microcolonies or empty spaces that become parts of the water channels (129).

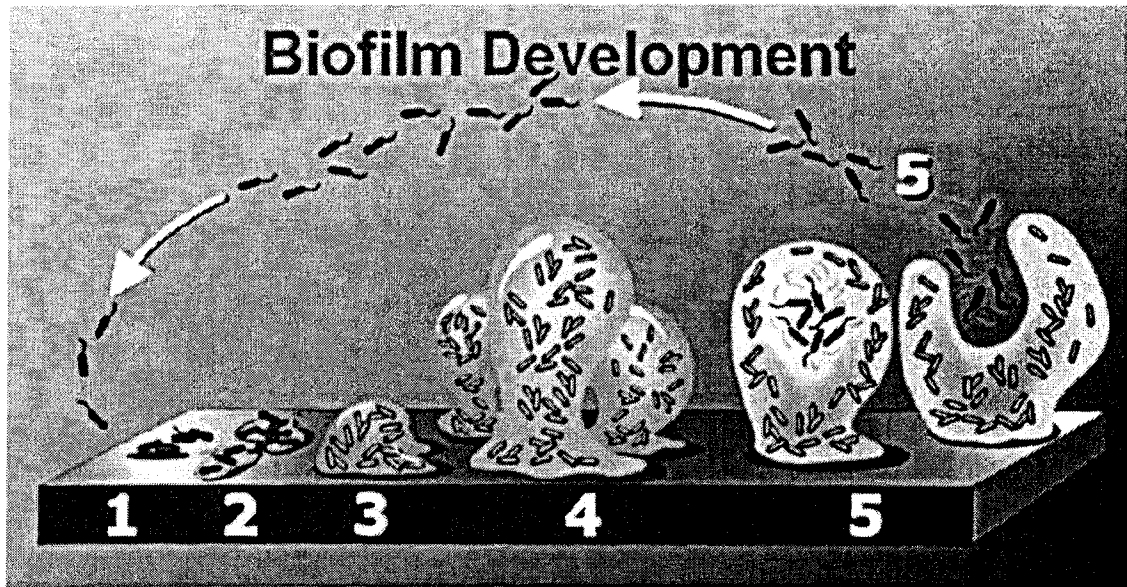


Figure 7. Developing of a biofilm as a five-stage process (129).

Plastic composite supports biofilm

The biofilm can be comprised of a single or multiple microbial species and can form on a range of biotic and abiotic surfaces. *In vitro*, the biofilms form readily on abiotic surfaces, such as wood chips, frittered glass particles, polyester foams, stainless steel wire, and various plastics (137). At Iowa State University, a material called plastic composite supports (PCS) was developed using a blend of polypropylene (PP) and agricultural, which were produced by high-temperature extrusion (107). PCS blends can be customized to enhance the biofilm formation. Polypropylene gives the PCS high mechanical stability and the ability to withstand sterilization temperatures and pH extremes. Ground soybean hulls is

the agricultural product of choice, because of its excellent water holding capacity (up to 191% of its dry weight) (108) and its ability to puff the extruded products. This generates a porous material with high surface area (72). The nutrients in PCS can be customized to support specific microbial requirements for the microorganism of choice (72). To protect micronutrients during high temperature extrusion, 5% (w/w) bovine albumin is added to all PCS blends (72). The PCS have been shown to stimulate microbial attachment (40), serve as a vehicle for slow release of nutrients during long term lactic acid fermentation (42), provide access of nutrients for microorganisms attached on the support surface (107), and help prevent wash-out of biomass from the reactor vessel (107).

The PCS have also been shown to stimulate biofilm formation and enhance productivity of specific end products. For example, Demirci et al. (41) showed that ethanol production by *Saccharomyces cerevisiae* could be increased up to 10 times when PCS were used during fermentation. Velazquez et al. (140) demonstrated an increase in lactic acid production by *Lactobacillus casei* in PCS-biofilm fed-repeated-batch reactors (2.45 g/1/h) compared to suspended-cell fed-repeated-batch reactors (1.75 g/1/h). Demirci et al. (40) demonstrated an increase in lactic acid production by up to five times over that of suspended culture when solid PCS were used. Urbance (137) evaluated the ability of succinic acid production in continuous and repeat-batch by *Actinobacillus succinogenes* PCS biofilm. Recently, Cotton et al. (28) demonstrated that the optimized PCS blend for *L. casei* stimulates biofilm formation and increase the lactic acid production in continuous fermentation with PCS tube attached to agitator shaft. Also, the biofilm thickness was controlled by agitation speed.

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Detoxification of Corn Stover and Corn Starch Pyrolysis Liquors by *Pseudomonas putida* and *Streptomyces setonii* Suspended Cells and PCS Biofilms

A paper to be submitted to Applied and Environmental Microbiology

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Abstract

Plant biomass can be liquefied into fermentable sugar (levoglucosan then to glucose) for production of ethanol, lactic acid, enzymes and more by a process called fast pyrolysis. *Pseudomonas putida* (ATCC 17484) and *Streptomyces setonii* 75Vi2 (ATCC 39116) were employed to detoxify diluted corn stover (Dcs) and diluted starch (Dst) pyrolysis liquors. Suspended cells illustrated limited detoxification ability of Dcs and Dst. *P. putida* and *S. setonii* PCS biofilms detoxified 10 and 25% (vol/vol) Dcs and Dst. Mixed culture PCS biofilms of *P. putida* and *S. setonii* partially detoxified 50% (vol/vol) and significantly detoxified 10 and 25% (vol/vol) Dcs and Dst. The detoxification process evaluation was via total phenols assay, change in UV absorbance spectra, GC-MS analysis, and a bioassay, which is the ability of *Lactobacillus casei* subsp. *raamosus* (ATCC 11443) to grow on a mixture of MRS media and detoxified Dcs or Dst (1:2, vol/vol) and produce lactic acid. Thus, a mixed cultured biofilm reactor with *P. putida* and *S. setonii* can be employed to detoxify pyrolysis liquors for further fermentation to value added products such as ethanol and lactic acid.

Introduction

Fast pyrolysis can liquefy agricultural residues into an alternative source of organic chemicals (6). Agricultural crop residues consist of lignocellulose, which are mainly cellulose, hemicellulose and lignin. When lignocellulosic material is rapidly heated in the absence of oxygen (a process known as fast pyrolysis) followed by cooling and condensing, a dark brown mobile liquid with a wide range of oxygenated organic chemicals compounds are produced. The liquid can be used in many static fuel applications for power generation and heating can be upgraded to conventional transport fuels and can be used to drive or manufacture a wide range of chemicals specialties and intermediates (6, 16). The fast pyrolysis process of cellulosic materials is the simplest method for biomass saccharification (30, 32). When the pyrolysis process is carried out under controlled conditions, a high yield of anhydrosugar is produced (29, 32). The anhydrosugar, primarily levoglucosan (1, 6-anhydro- β -D-glucopyranose), is dehydrated glucose, which contains an intermolecular glucosidic bond, and which can serve directly or indirectly as a feedstock for the fermentation industries to produce both fuels and chemicals (7, 32, 51).

Several studies mentioned the potential of utilizing the levoglucosan as a precursor for the synthesis of rare sugars, herbicidal derivatives or as substrate for microbial fermentations (8, 17, 26). Levoglucosan can also be used indirectly through hydrolyzing with mild acid to produce glucose (29) or directly metabolized by eukaryotic and prokaryotic microorganisms (30). The eukaryotic microorganisms, which have levoglucosan kinase, convert levoglucosan to glucose-6-phosphate, while prokaryotic microorganisms metabolize levoglucosan through multiple steps reaction. The enzyme (levoglucosan dehydrogenase) catalyzes the dehydrogenation of levoglucosan to 3-keto levoglucosan, then to 3-keto glucose,

and finally to D-glucose by using NAD^+ as an electron acceptor (30). Unfortunately, not all eukaryotic or prokaryotic can utilize the levoglucosan, which is generated from the biomass pyrolysis process. Numerous compounds, such as phenols, benzene, furan, fufuryl derivatives and many other oxygenated compounds are generated during biomass pyrolysis (16, 28) These compounds are toxic or inhibitors to microbial growth (36). Therefore, post-pyrolysis treatments such as activated carbon and solvents extraction are usually required for post-pyrolysis clean up (29, 37, 53). However, these methods result in waste generation with negative environmental and economic impact including some loss of levoglucosan. The use of biological treatments to detoxify chemical compounds seems promising due to being cost-effective and environmentally compatible.

The use of specialized or selected microorganisms to degrade toxic compounds has received considerable interest, since each microorganism is known to possess different detoxification mechanisms. Most bacteria degraded toxic compounds via enzymes induced by the toxic compound (8) and these enzymes can degrade other toxic compounds (38). Also, *Pseudomonas* strains were found capable of cometabolic degradation of aromatic compounds and one of the compounds at least being used as a carbon source (5). Bacteria can degrade many toxic compounds, but the degradation of a specific compound in a mixture is impacted by other substituents of the mixture (38). The defined mixed culture is an alternative to uncharacterized mixed culture which may produce undesirable toxic metabolites that inhibit the growth. The defined mixed culture could degrade compounds in mixture through cooperative activity (3). The mixed culture also exhibited a good capacity to remove aromatic compounds when they are more water-soluble (45).

Moreover, the organic-solvent-tolerant bacteria such as *Pseudomonas putida* are relatively novel groups of microorganisms (39). They overcome the toxic and destructive effects of organic solvents by the presence of various adaptive mechanisms (20, 37). It was assumed that Gram-negative bacteria are better equipped to cope with solvent induced shock (23) because it have an outer membrane compared to the single cytoplasmic membrane in Gram-positive bacteria (39). Yet, strains of Gram-positive bacteria like *Rhodococcus* tolerant to benzene have been reported (31). Organic solvents are known to be extremely toxic to microbial cells (39). Solvents are known to accumulate in and disrupt the bacteria cell membrane affecting the structural and functional integrity of the microbial cells (37, 39). On the other hand, most of organic-solvent-tolerant bacteria, such as *Pseudomonas* develop biofilm (27, 42, 43). The biofilm-associated cells are showed more resistant to many toxic substances such as antibiotics, chlorine, and detergents (10). The reasons for resistance might be due to the decreased bacterial growth rate, cell signaling, decrease the diffusion into the biofilm, and forming biofilm-specific substance such as exopolysaccharide (EPS) (13, 50).

The biofilm is considered a natural form of immobilized cells attached to a variety of surfaces rather than being swept away by the water current (35). However, when the biofilm are utilized in fermentation, attention must be paid to the movement between substrate and cell so that it dose not become rate limiting (12). At Iowa State University (ISU), we developed a novel biofilm support called plastic composite supports (PCS) tubes, which can be customized for the microorganism of choice to produce a PCS biofilm (21, 35, 46), and can be cut at angle and fixed to the agitator shaft in a stir tank bioreactor to allow fermentation media to flow through the inside of the PCS tubes (11). The PCS tubes have

been shown to stimulate biofilm formation and enhance productivity of end products (11, 14, 47).

This research aims to demonstrate the ability of suspended cell and PCS biofilm as biological detoxification process of corn stover and corn starch pyrolysis liquor. The detoxification process is referred to remove or reduce the toxic compounds of the pyrolysis liquors as the results of the microbial growth. Two microorganisms *P. putida* and *Streptomyces setonii*, which are unable to utilize levoglucosan, but have the ability to utilize numerous aromatic compounds (2, 3, 9, 33, 38, 40) were employed as suspended-cell and PCS-biofilm batch cultures to remove or reduce toxicity of pyrolysis liquors. The mixed culture PCS biofilm is a novel technique to detoxify different concentration of diluted pyrolysis liquors in computer controlled bench top fermentations.

Material and Methods

Microorganisms

Streptomyces setonii 75Vi2 (ATCC 39116), *Pseudomonas putida* (ATCC 17484) and *Lactobacillus casei* subsp. *ramosus* (ATCC 11443) were obtained from American Type Culture Collection (Manassas, Virginia). *P. putida* was maintained on agar slants containing 1.0 g /l of beef extract, 2.0 g /l of yeast extract, 5.0 g /l of peptone, 5.0 g/l of NaCl, 0.45 g /l of KH_2PO_4 , 2.39 g /l of Na_2HPO_4 and 15.0 g /l of agar (Sigma Chemical Company, St. Louis, MO) and stored at 4°C. The initial pH of the medium was 6.8 (18). *S. setonii* was maintained at 4°C as sporulated culture on agar slants of a rich organic media, such as yeast extract-malt agar (34). *L. casei* was maintained as freeze-dried culture and as working

culture by monthly transfers in MRS broth (Difco Laboratories, Detroit, MI) and stored at 4°C (15).

Chemicals

Yeast extract (Ardamine Z) was obtained from Sensient Flavor (Juneau, WI). Glucose (Cerelese) was obtained from International Ingredients (St. Louis, MO). 2-methoxy-5-methylphenol, 2,6-dimethoxy phenol, 1,2,4-trimethoxybenzene, 2(5H)furanone, and 1,6-anhydro- β -D-glucopyranose (levoglucosan) were obtained from Sigma Chemical Company (St. Louis, MO).

Pyrolysis liquors preparation

The pyrolysis process was carried out in a pilot-scale fluidized pyrolyzer (Figure 1) at Iowa State University. The reactor consists of a 15-cm dia. bubbling fluidized bed operated at 400 to 600°C. It processes 2.5 to 5.0 kg/h of biomass to produce liter quantities of pyrolysate. Heat to drive pyrolysis is provided by burning pyrolytic gas or natural gas in an external burner and directing this hot gas through a heat exchange jacket surrounding the pyrolytic reactor. Recirculated pyrolytic gas serves as the fluidization agent for the bubbling bed, thus avoiding addition of air to the reactor, which would reduce pyrolysate yield. Products of pyrolysis include gas (a flammable mixture consisting of hydrogen, carbon monoxide, carbon dioxide, and some non-condensable hydrocarbons), particulate matter (ash and char), and condensable vapors. Particulate matter is removed by insulated cyclones designed to keep the gas stream at high temperature. The gases and vapors then enter a series of four, water-cooled, shell-and-tube heat exchanges designed to condense water and organic

vapors, which forms the pyrolysate used in the fermentation trials. In this research, the pyrolysate was prepared from corn stover which is residual after harvest. It is composed of 50% cellulose with some hemicelluloses and lignin (6). Commercial corn starch available at local grocery stores could be subjected to a variety of chemical modifications that aimed to improve functionality for practical applications (28). The pyrolysate which generated in this pyrolysis process were called corn stover pyrolysis liquor and corn starch pyrolysis liquor. The pyrolysis liquors are brown color, a distinctive odor, and contain significant yields of levoglucosan and monosaccharides. *P. putida* and *S. setonii* are unable to grow on these substrates. To prepare both pyrolysis liquors for fermentation they were diluted with deionized water at 1: 2 ratio, vigorously mixed, then stored overnight at 4°C. Insoluble precipitates (IP) were removed by centrifugation at 10,000 x g for 15 min at 10°C. The supernatants were neutralized with CaCO₃ then centrifuged at 15,000 x g for 20 min at 10°C. The supernatant of diluted corn stover (Dcs) and diluted corn starch (Dst) pyrolysis liquors were filter sterilized by 0.45 µm PTFE membranes to minimize any changes in chemical composition that might result from heat sterilization.

Growth on pyrolysis liquors

The ability of *P. putida*, *S. setonii* and *L. casei* to grow and metabolize the toxic compounds in Dcs and Dst was carried out in 250 ml shake-flask cultures and in plates. Each microorganism was inoculated in replicates of three. For *P. putida* 50 ml of medium consisted of 420 mg /l of KH₂PO₄, 375 mg /l of K₂HPO₄, 244 mg /l of (NH₄)₂SO₄, 30 mg /l of NaCl, 30 mg /l of CaCl₂ · 2H₂O, 61.4 mg /l of MgSO₂ · 7H₂O and 4.7 mg /l of FeCl₂ · 4H₂O (18) and 5% (vol/vol) of filter sterilized Dcs or Dst. This medium was inoculated with 0.5 ml

of (0.5 A_{620}) 18 hr *P. putida* culture incubated in same medium but with 20 g/l glucose instead of 5% (vol/vol) Dcs or Dst.

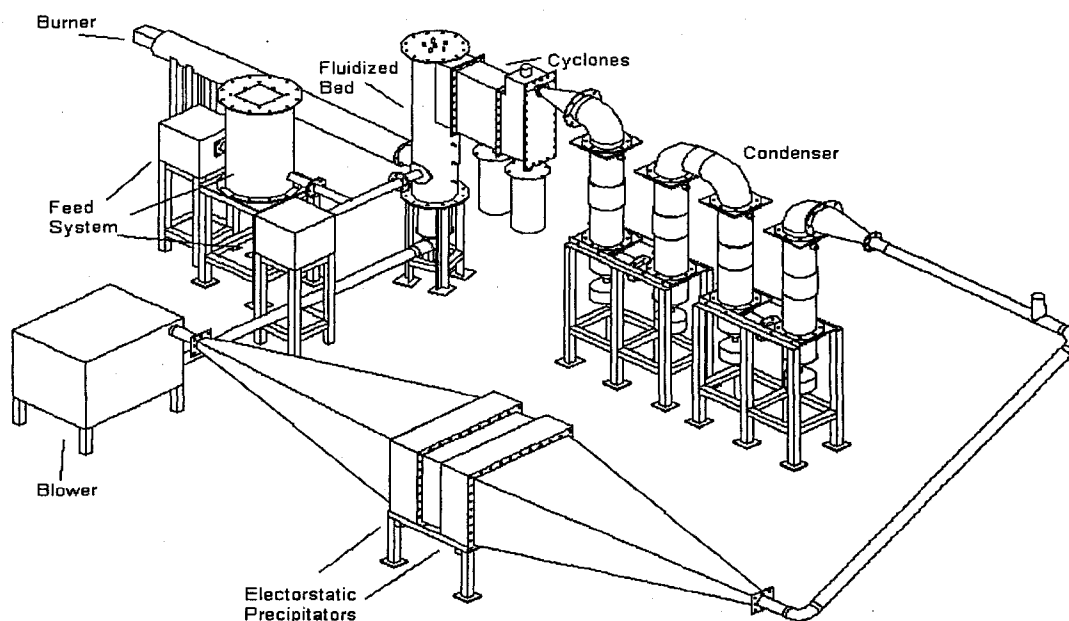


Figure 1. Pyrolysis pilot plan.

S. setonii culture spores grown on yeast extract-malt agar slant were used for inoculating 50 ml of medium consisting of 0.005% (wt/vol) of yeast extract (Difco Laboratories) and mineral salts solution [5.3 g/l of Na_2HPO_2 , 1.98 g/l of KH_2PO_2 , 1 g/l of $(\text{NH}_2)_2\text{SO}_4$, 0.2 g/l of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g/l of NaCl , 0.05 g/l of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$], 100 ml/l trace elements (34) and 5% (vol/vol) of filter sterilized Dcs or Dst. For *L. casei* 0.5 ml (0.5 A_{620}) of 18 hr culture grown on MRS broth was inoculated in 50 ml medium consisting of 10 g/l of yeast extract (Ardamine Z), 6 g/l of $\text{MgCl}_2 \cdot 7\text{H}_2\text{O}$, 0.3 g/l of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 5 g/l of

KH_2PO_4 and 5 g /l of K_2HPO_4 (15) and 5% (vol/vol) of filter sterilized Dcs or Dst. The inoculated flasks were incubated shaking for 72 hr at 30°C for *P. putida* (18) and at 37°C for *S. setonii* and *L. casei* (15, 34). The ability of the microorganisms to grow on filter sterilized Dcs or Dst was also determined on agar plates in which 15 g/l of agar was added to each media.

Growing on levoglucosan

The ability of *P. putida*, *S. setonii* and *L. casei* to metabolize levoglucosan was investigated using the same previous media and procedure for each bacterium, except 0.5% (wt/vol) filter sterilized levoglucosan was added instead of diluted pyrolysis liquor Dcs or Dst. The control cultures were with glucose instead of Dcs, Dst or levoglucosan. Growth was determined visually on plates and spectrophotometer (cell density) by measuring A_{620} of shake flasks cultures.

Detoxification with suspend cells

In order to determine the ability of *S. setonii* and *P. putida* suspended cells to detoxify high concentration of the filter sterilized pyrolysis liquor Dcs and Dst 10, 25, and 50% (vol/vol) were added to a medium consisting of 1.0% (wt/vol) of yeast extract, 10.0 g /l of CaCO_3 and pH 6.5. Each flask culture was inoculated with a fresh culture of 24 hr *S. setonii* or 18 hr *P. putida* (0.5/ml at A_{620}) and evaluated in a replicate of three for each concentration of Dcs and Dst. All inoculated flasks were incubated shaking at 125 rpm for 10 days at 30°C for *P. putida* and 37°C for *S. setonii*. The control flasks were not inoculated.

Detoxification with PCS biofilms

The biofilm culture of *P. putida*, *S. setonii* or mix culture was developed on PCS tubes attached to the agitator shaft which it stimulates biofilm development. The PCS blend for this research contained 50% (wt/wt) of polypropylene (PP) to give the PCS high mechanical stability and the ability to withstand sterilization temperatures and high pH, 40% (wt/wt) ground dried soybean hull (Cargill Soy Processing Plant, Iowa Falls, IA) to create a porous surface, 5% (wt/wt) dried bovine albumin (American Protein Corp., Ames, IA) to protect micronutrients during high temperature extrusions (21), 5% (wt/wt) yeast extract (Ardamine Z) and mineral salts (0.6 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g/l KH_2PO_4 , 1 g/l Sodium acetate, 0.03 g/l $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ and 0.5 g/l K_2HPO_4) to reduce PCS hydrophobicity (21). The PCS was produced according to Ho et al. (21). These dry ingredients were mixed in separate container prior to being poured into extruder hopper. The twin screw co-rotating Bra bender PL2000 extruder (model CTSE-V; C. W. Bra bender Instruments, Inc; South Hackensack, N. J.) was operated at a rate of 11 rpm, barrel temperatures of 200, 220, and 200°C, and a die temperature of 167°C to form a continuous tube. Composite supports with a wall thickness of 3.5 mm and an outer diameter of 10.5 mm were cut into 10 cm lengths and end cut at an angle to allow the media to flow through the inside of tubes. Six PCS tubes were stacked in rows of two parallel tubes then bound to the agitator shaft in a grid-like fashion (11) (Figure 2).

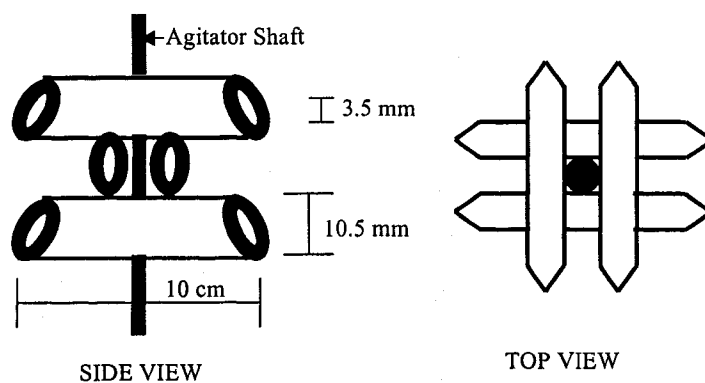


Figure 2. PCS tubes bound to agitator shaft.

Reactor

A computer controlled New Brunswick Bioflo 3000 (Edison, NJ) benchtop fermentor equipped with pH, temperature, agitation and oxygen dissolved controls was employed with batch and continuous cultures. The 1.2 L vessel was equipped with filtered sterilized air in and out, alkali, acid, medium addition, and broth removal ports (Figure 3). To control pH acid and alkali were added from graduated burettes, which were refilled aseptically from reservoirs with 1 N HCl and 1 N NaOH. The dissolved oxygen (DO_2) and pH controls allowed rapid determination of biological activity. The broth removal port was connected with two branched lines to withdraw a sample and to draw off all culture media from the vessel. On the side of the vessel a removal port equipped with a break was used to run continuous culture. The reactor was sterilized with water in the autoclave for 1.25 hr at 121°C . After sterilization, media was used to dilute out water at a dilution rate of 0.6 h^{-1} (11).

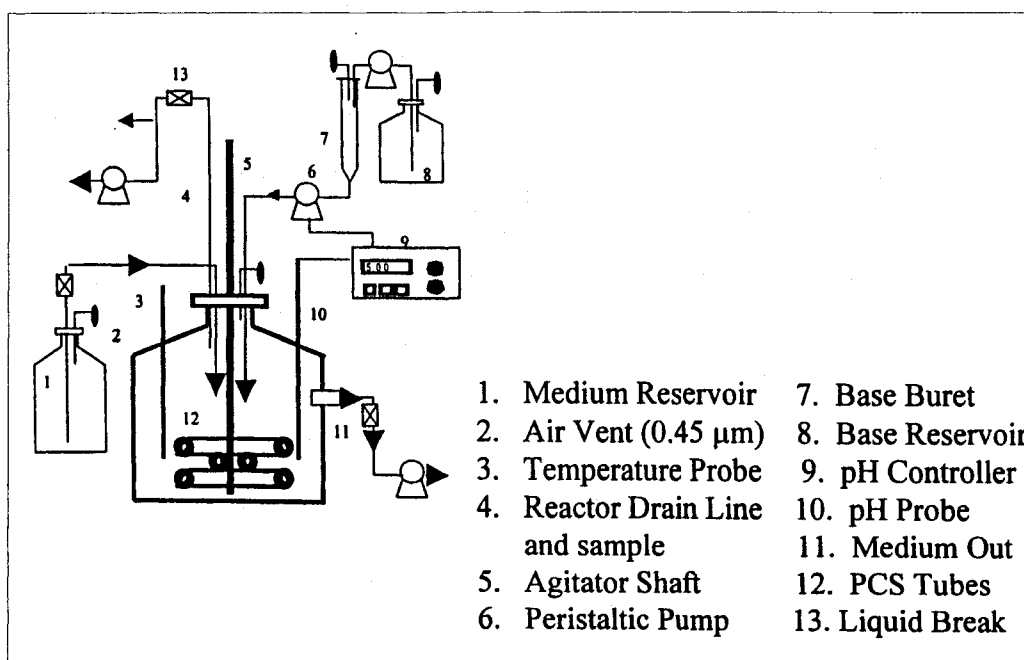


Figure 3. Bioreactor Design.

Medium

The same medium, which was used in the detoxification Dcs and Dst with suspend cell was modified to work with computer control bench top fermentor with PCS attached to agitator shaft to develop biofilm and that included omits the CaCO_3 from the medium and controlled the pH by 1 N NaOH and 1 N HCl. The biofilm culture developed via batch and continuous culture and the medium consisted of 1% (wt/vol) of yeast extract (Ardamine Z) and 20 g /l of glucose. The detoxification medium consisted of 10 g /l of yeast extract (Ardamine Z) and 10, 25 or 50% (vol/vol) of filter sterilized pyrolysis liquors. Biofilm culture media and detoxify culture media were prepared in 90-L batches, sterilized in a B-Braun 100-D fermentor (Allentown, PA) with continuous agitation for 25 min at 121°C , and adjusted to pH 6.5 with sterile 3 N HCl. The sterilized media was aseptically transferred into

two sterilized 50-L carboys equipped with a carboy filling port, a medium delivery line with a liquid break, and an air vent capped with a 0.45 μm air filter for storage.

Optimal pH and temperature

Since the CaCO_3 was used to control the pH in suspend cell shake-flask cultures a repeated-batch suspend cell culture was employed with benchtop fermentor to determine optimum pH and temperature for the *P. putida* and the *S. setonii*. The growth of *P. putida* and *S. setonii* was evaluated in batch culture media at pH 6.0, 6.5, 7.0, and 7.5 with constant agitation (100 rpm) and 30 and 37°C with overnight incubation for each treatment respectively. In the beginning of each treatment the fermentor was inoculated either with 5 ml suspension of 24 hr *S. setonii* culture spores or with 5 ml (0.5/ml at A_{620}) of 18 hr *P. putida* culture. The optimal growth was determined via absorbance at 620 nm.

P. putida biofilm

P. putida biofilm was developed on the PCS (Figure 2) via batch and continuous cultures using the previously described media at pH 6.5, constant agitation (100 rpm), and 30°C incubation temperature. The fermentor was incubated as batch culture with 5 ml (0.5/ml at A_{620}) 18 hr inoculums of *P. putida* previously prepared. The continuous culture was started after 24 hr with high dilution rate of 1.4 h^{-1} for 8 to 10 hr, then the fermentor was incubated again as batch culture overnight and low dilution rate 0.1 h^{-1} was run for 11 days.

S. setonii biofilm

Fresh PCS tubes were fixed to the agitator shaft in the reactor (Figure 2) and all the setting and sterilizing steps were performed again. The fermentor was incubated overnight with previous described media and inoculated with 5 ml suspension of 24 hr *S. setonii* spores grown on yeast extract-malt agar slant. The *S. setonii* biofilm was developed via continuous culture by increasing the dilution rates gradually 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 to 1.4h^{-1} . The continuous culture was performed with pH 6.5 and constant agitation (100 rpm) for 7 days.

Mixed culture biofilm

Fresh PCS tubes were fixed to the agitator shaft in the reactor (Figure 2) and the same procedures described for single cultures were followed. The *S. setonii* biofilm was allowed to develop first as described previously then the reactor was drained. Fresh media was added to the reactor and inoculated with 5 ml ($0.5/\text{ml}$ at A_{620}) *P. putida* then the batch and continuous culture were performed as described previously. Daily sample was collected and examined by Gram stain. After 18 days the reactor was developed mixed biofilm cultures. The series steps to develop mixed culture biofilm were summarized in figure 4.

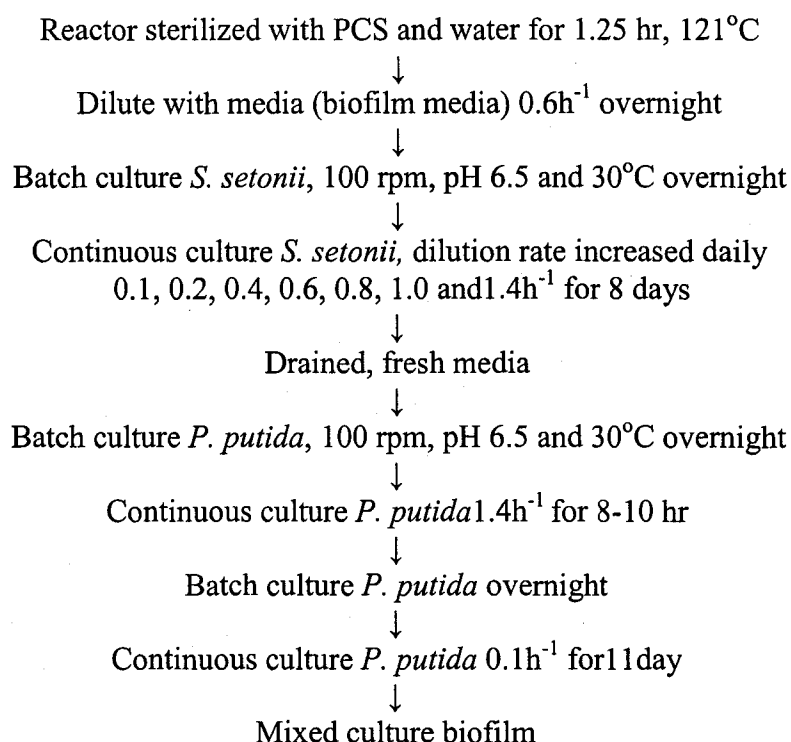


Figure 4. Series of steps summarized setting, sterilizing and developing mixed culture biofilm.

Biofilms cultures detoxification

The reactor developed with *P. putida* biofilm culture was supplied with detoxification media. Three concentration 10, 25, and 50% (vol/vol) of filter sterilized Dcs and Dst were evaluated. The detoxification process was run as batch culture one liter total volume with pH 6.5, constant agitation (100 rpm) at 30°C. Sample was recovered on day zero (control), four and ten. The detoxification process was monitored by measuring changes in DO₂ and pH. After ten days, the reactor was drained and the detoxified effluents were evaluated. The reactor was refilled with fresh medium and same concentration of Dcs for reevaluation. The previous steps were performed with 10, 25, 50% (vol/vol) Dcs and Dst. Prior to the addition

of a new concentration of Dcs or Dst the reactor was drained and refilled with fresh media and run as continuous culture for 2-3 hrs at dilution rate 0.4h^{-1} to remove any residual spent medium that might be remaining. The same procedures and the concentrations of filter sterilized Dcs and Dst were performed with *S. setonii* biofilm and mixed culture biofilm (Figure 5). In detoxification with mixed culture biofilm samples were recovered after each run and examined with Gram stain to insure the mixed culture biofilm.

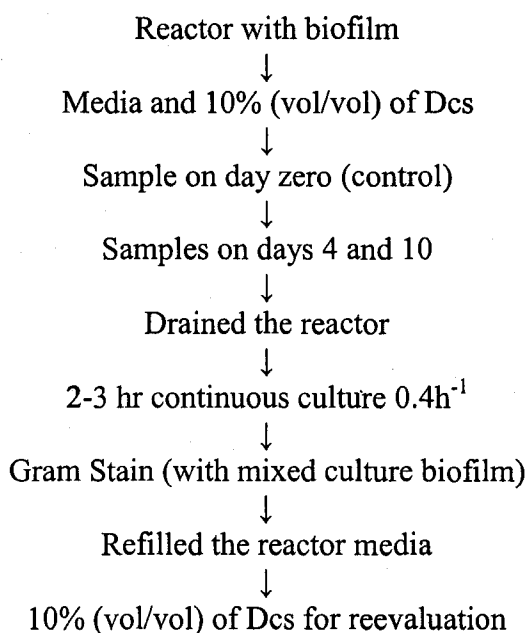


Figure 5. Series of steps to detoxify 10% (vol/vol) Dcs.

Samples analysis

UV absorption spectra

The change between detoxified and non-detoxified Dcs and Dst samples were monitored by UV absorption spectrum. The absorption spectrum was determined in 200-300

nm via Beckman UD 40 from Beckman Instruments, Inc. (Fullerton, CA).

GC-MS analysis

Twenty five ml of detoxified and non-detoxified Dcs and Dst samples were acidified to pH 4.0 with HCl and then extracted three times with ethyl acetate, three times with ethyl acetate/acetone 2:1 and three times with ethyl ether (25). The pooled organic phases were dehydrated with anhydrous sodium sulphate and the solvents were removed. The dried extract was resuspended in 25 ml ethyl acetate and filtered. The analysis was performed by using Agilent 6890 series GC system with Agilent autosampler 7683 series injector and equipped with micromass GCT mass spectrometer, and column of JW DB.5 MS (30 m by 0.25 mm by 0.25 μ m; Agilent). The sample injected as split ratio 1 to 100. For the quantitation analysis, five compounds in Dcs samples including phenol, 2-methoxy-5-methylphenol, 2,6-dimethoxy phenol, 1,2,4-trimethoxybenzene, and 1,6-anhydro- β -D-glucopyranose (levoglucosan) and two compounds in Dst including 2(5H) furanone and levoglucosan were used as standard to perform quantitative analysis in detoxified and non-detoxified Dcs and Dst samples. The levoglucosan is partially dissolved in ethyl acetate. For levoglucosan GC-MS quantitative analysis, 25 ml of detoxified and non-detoxified Dcs and Dst were dehydrated and then the dried extract was resuspended in 25 ml methanol.

Total phenols

Phenolic compounds were extracted as above for GC-MS analysis then resuspended in 25 mM phosphate buffer (pH 6.0) instead of ethyl acetate. The phenolic assay is based on the oxidation of phenolate ion where ferric ions are reduced to the ferrous state which was

detected by the formation of the Prussian blue complex ($\text{Fe}_4[\text{Fe}(\text{CN})_6]_3$) with a potassium ferricyanide-containing reagent (49). The reagents of the assay were ferric chloride reagent, which was prepared by mixing 0.1 M solution of ferric chloride (FeCl_3) in 0.1 M hydrochloric acid, and potassium ferricyanide reagent, which was 0.008 M $\text{K}_3\text{Fe}(\text{CN})_6$ in deionized water (49). The assay mixture contained 25 ml of deionized water, 250 μl of sample, 3 ml of ferric chloride reagent and 3 ml of potassium ferricyanide reagent. The sample absorbance was measured at 720 nm. The phenolic contents in the samples were expressed as syringic acid equivalents (10 $\mu\text{g ml}^{-1}$ gives an optical density of 0.377/ml at 760 nm) (1).

Unfortunately, any substances capable of being oxidized by the reagents of the total phenolic assay will reduced (colored) forms of the reagents and appear as a phenolic. For example, ascorbic acid is one commonly occurring non-phenolic capable of doing this (49). Similar interfering result has been found with Dst (data not shown) although no phenolic compounds exist based on the GC-MS analysis. Therefore, the total phenols assay was performed only with Dcs samples.

Bioassay

The samples collected for the bioassay were centrifuged at $10.000 \times g$ for 20 min at 20°C and then filter sterilized with a 0.45 μm filter membrane. The bioassay was carried out in 250-ml flasks by mixing 2:1 (vol/vol) of detoxified Dcs or Dst and MRS media. The flasks were inoculated with 0.5 ml (0.5/ml at A_{620}) of 18 hr *L. casei* culture and incubated at 37°C as a static culture for 24 hr. The control was incubated under the same conditions with different concentrations of non-detoxified Dcs or Dst. The samples were analyzed for cell

density by absorbance at 620 nm using a spectronic 20 and lactic acid production by using Hewlett Packard (San Fernando, CA) high-pressure liquid chromatograph (HPLC) 1100 equipped with Waters model 2410 refractive index detector, column heater, autosampler, and computer controller. Lactic acid was separated on a Bio-Rad Aminex HPX-87H column (300 x 7.8 mm) (Bio-Rad Chemical Division, Richmond, CA) with 0.12 N sulfuric acid as the mobile phase at a flow rate of 0.8 ml/min with a 20 μ l injection volume and a 65°C column temperature.

Results

Growth on pyrolysis liquors

P. putida and *S. setonii* grew on 5.0% (vol/vol) of filter sterilize Dcs and Dst in plates and flasks, but *L. casei* did not grow (Table 1). The growth on plates was monitored visually and recorded as either growth or no growth. The *P. putida* and *S. setonii* colonies were visible after 26 and 36 hr of incubation, respectively. The shake-flasks cultures were monitored through cell concentration A_{620} . After 72 hr the *P. putida* A_{620} was 1.8 and 1.5 while *S. setonii* A_{620} was 2.5 and 1.5 and the control A_{620} was 3.9 and 3.5 for Dcs and Dst, respectively. The *L. casei* did not grow with 5.0% (vol/vol) Dcs or Dst (Table 1). None of the bacteria grew on the plates or in the flasks cultures supplied with 0.5% (wt/vol) of filter sterilized levoglucosan as sole carbon source.

Table 1. Abilities of the bacteria to grow on the Dcs, Dst and levoglucosan in shake-flask cultures and agar plates^a.

Bacteria	Cell density (A_{620}) in shake-flask cultures				Plates		
	Dcs ^b	Dst ^c	levo ^d	C ^e	Dcs	Dst	levo
<i>P. putida</i>	1.8	1.5	- ^f	3.9	+	+	-
<i>S. setonii</i>	2.5	1.5	-	3.5	+	+	-
<i>L. casei</i>	0.0	0.0	-	4.3	-	-	-

^a Each value represents an average of three replicates

^b Growth media with 5.0% (vol/vol) of filter sterilized diluted corn stover

^c Growth media with 5.0% (vol/vol) of filter sterilized diluted corn starch

^d Growth media with 0.5% (vol/vol) levoglucosan

^e (Control) growth media with glucose and no Dcs or Dst

^f Indicate growth or no growth

Detoxification with suspended cell

S. setonii and *P. putida* grew on media containing yeast extract and CaCO_3 and different concentrations of Dcs and Dst. The *S. setonii* and *P. putida* suspend cells grew on 10% (vol/vol) Dcs and Dst (Table 2). With 10% (vol/vol) Dcs the A_{620} for *P. putida* was 1.9 and for *S. setonii* 2.3. With 10% (vol/vol) Dst the A_{620} was 1.1 for *P. putida* and 1.5 for *S. setonii*. The growing on 25% (vol/vol) Dcs and Dst were 0.3 and 0.1 A_{620} for *S. setonii*, but *P. putida* did not grow. Neither *S. setonii* nor *P. putida* grew on 50% (vol/vol) of Dcs and Dst (Table 2). Result of growing the *P. putida* and *S. setonii* on the 10 and 25% (vol/vol) Dcs and Dst removed toxic compounds which was illustrated *L. casei* growth and production of lactic acid during the bioassay (Table 3).

Table 2. Suspend cells bacterial growth with different concentrations of Dcs and Dst in shake-flask cultures^a.

Bacteria	Cell density (A_{620}) in shake-flask cultures						
	Control ^b	Dcs ^c			Dst ^d		
		10% (vol/vol)	25% (vol/vol)	50% (vol/vol)	10% (vol/vol)	25% (vol/vol)	50% (vol/vol)
<i>P. putida</i>	- ^e	1.9	-	-	1.1	-	-
<i>S. setonii</i>	-	2.3	0.3	-	1.5	0.1	-

^a Each number represents the average of three replicate

^b Control (represent all concentrations of Dcs and Dst without inoculation)

^c Diluted corn stover

^d Diluted corn starch

^e Indicated no growth

Table 3. *L. casei* growth on detoxified Dcs and Dst by *P. putida*, and *S. setonii* suspended cell and mixed cultures biofilms^a.

Detoxified media by	Control ^b		Detoxified Dcs ^c						Detoxified Dst ^d					
			10% (vol/vol)		25% (vol/vol)		50% (vol/vol)		10% (vol/vol)		25% (vol/vol)		50% (vol/vol)	
	A_{620}	LA ^e g/l	A_{620}	LA g/l	A_{620}	LA g/l	A_{620}	LA g/l	A_{620}	LA g/l	A_{620}	LA g/l	A_{620}	LA g/l
<i>P. putida</i> ^f	-	- ^g	2.1	3.2	0.04	1.8	-	-	2.2	3.1	-	-	-	-
<i>S. setonii</i>	-	-	1.2	5.3	1.0	3.4	-	-	2.5	4.9	-	-	-	-
<i>P. putida</i> ^h	-	-	2.1	7.2	1.9	5.1	-	-	2.1	6.1	2.2	6.0	0.1	-
<i>S. setonii</i>	-	-	1.6	8.3	2.1	7.2	-	-	2.6	6.3	3.4	6.2	-	-
Mixed culture	-	-	2.6	17.7	3.6	16.3	2.9	9.8	3.2	17.9	4.4	13.4	2.2	6.8

^a Each number represent average of three replicates

^b Control, mixture of MRS media and non detoxify media inoculated with *L. casei*

^c Detoxified corn stover pyrolysis liquor

^d Detoxified starch pyrolysis liquor

^e Lactic acid production

^f Suspended cell

^g Indicated no growth or no lactic acid production

^h Biofilms

Biofilm formation

The optimum conditions for *P. putida* and *S. setonii* to develop biofilm was determined in repeated-batch suspend cell culture, which showed that both *P. putida* and *S. setonii* can grow with a wide range of pH. The optimal pH for *P. putida* was 6.5, while *S. setonii* grew well in pH 6.5 and 7.2. The *P. putida* did not grow well at 37°C but both *P. putida* and *S. setonii* grew well at 30°C. Therefore, pH 6.5 and 30°C were employed on PCS biofilm reactors. The *P. putida* PCS biofilm was developed by changing the dilution rate from 1.4 h⁻¹, running from 8 to 10 hr after 24 hr of batch culture, to 0.1 h⁻¹ for 11 days. Light biofilm was noticed on the PCS after 7 days and thick biofilm was visually confirmed after 11 days. *S. setonii* PCS biofilm was defined on the PCS after 2 days at a dilution rate of 0.2 h⁻¹ and it increased visibly with increasing dilution rates. The thick biofilm, which covered all the PCS, was recorded on the 7th day with the dilution rate of 1.4 h⁻¹. Mixed culture biofilm consisted of *S. setonii* and *P. putida* biofilm. The *S. setonii* biofilm was cultured first on PCS followed by *P. putida* biofilm. Each biofilm developed through the same previous conditions (Figure 4). Gram stain of samples collected from the biofilm mixed culture media illustrated the presence filamentous Gram positive and Gram negative rods, for *S. setonii* and *P. putida*, respectively. The mixed biofilm was thick and viscous.

Detoxification with biofilm

Continuous of detoxification process was monitored by measuring changes in DO₂ and pH. During the detoxification of 10% (vol/vol) Dcs and Dst the DO₂ decreased rapidly within the first two days and then remained at a low level till the end of the study (Figure 6). Whereas, with the 25% (vol/vol) Dcs and Dst the DO₂ remained in high levels till the day six

and then it decreased rapidly with alkali (1 N NaOH) consumption more after day 7 of the detoxification (Figure 6). The bacterial biofilm cultures were effective in Dcs and Dst detoxification hence removing the toxic compounds from the Dcs and Dst which enable the *L. casei* to grow and produce the lactic acid in the bioassay (Table 3). The *S. setonii* and *P. putida* biofilm cultures detoxified 10 and 25% (vol/vol) Dcs and Dst. However, neither *S. setonii* nor *P. putida* biofilm were able to detoxify 50% (vol/vol) Dcs or Dst, whereas mixed culture biofilm did. However, the bioassay findings with detoxification 50% (vol/vol) Dcs and Dst (Table 3) were lower than those recorded with detoxification 10 and 25% (vol/vol) Dcs and Dst.

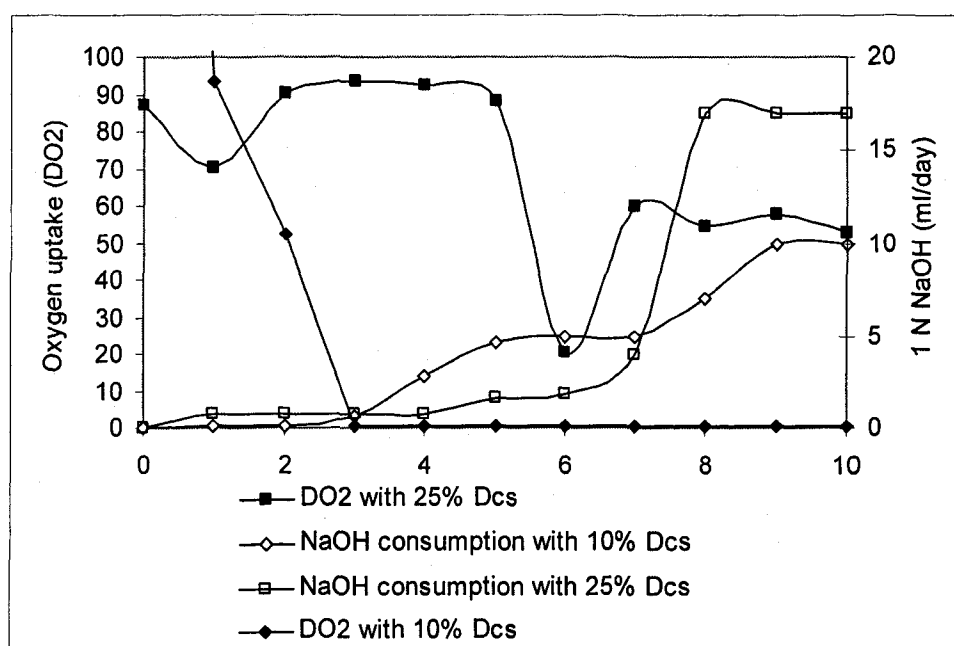


Figure 6. Oxygen uptake in bioreactor during the detoxification of 10 and 25% (vol/vol) Dcs by *P. putida* PCS biofilm.

Reduction in toxic compounds

Reduction of the toxic compounds was monitored over all by the change in UV absorption of the Dcs and Dst amended media. The UV spectrum showed that the change in detoxified 10% (vol/vol) Dcs was less significant (Figure 7) comparing to the sample detoxified with biofilms. The detoxified 25% (vol/vol) Dcs and Dst showed a significant decrease in $A_{200-300}$ spectra especially with the mixed culture biofilm (Figure 8).

Phenolic compounds reduction

The phenolic compounds reduction was determine in the Dcs detoxified samples. The reduction of phenolic compounds by *P. putida* or *S. setonii* suspended cell was less significant than the biofilms. The *P. putida* suspended cells reduce the phenolic compounds in 10, 25 and 50% (vol/vol) Dcs to 0.03, 0.2 and 0.5 mg/ml. While *S. setonii* suspended cell reduced the phenolic compounds to 0.04, 0.2 and 0.5 mg/ml. The *P. putida* biofilm reduce the phenolic compounds in 10, 25 and 50% (vol/vol) Dcs to 0.0, 0.1 and 0.3 mg/ml, the *S. setonii* biofilm while reduced it to 0.0, 0.1 and 0.4 mg/ml. The mixed culture biofilm removed the phenolic compounds from the 10 and 25% (vol/vol) Dcs but it remained 0.2 mg/ml in 50% (vol/vol) Dcs (Figure 9).

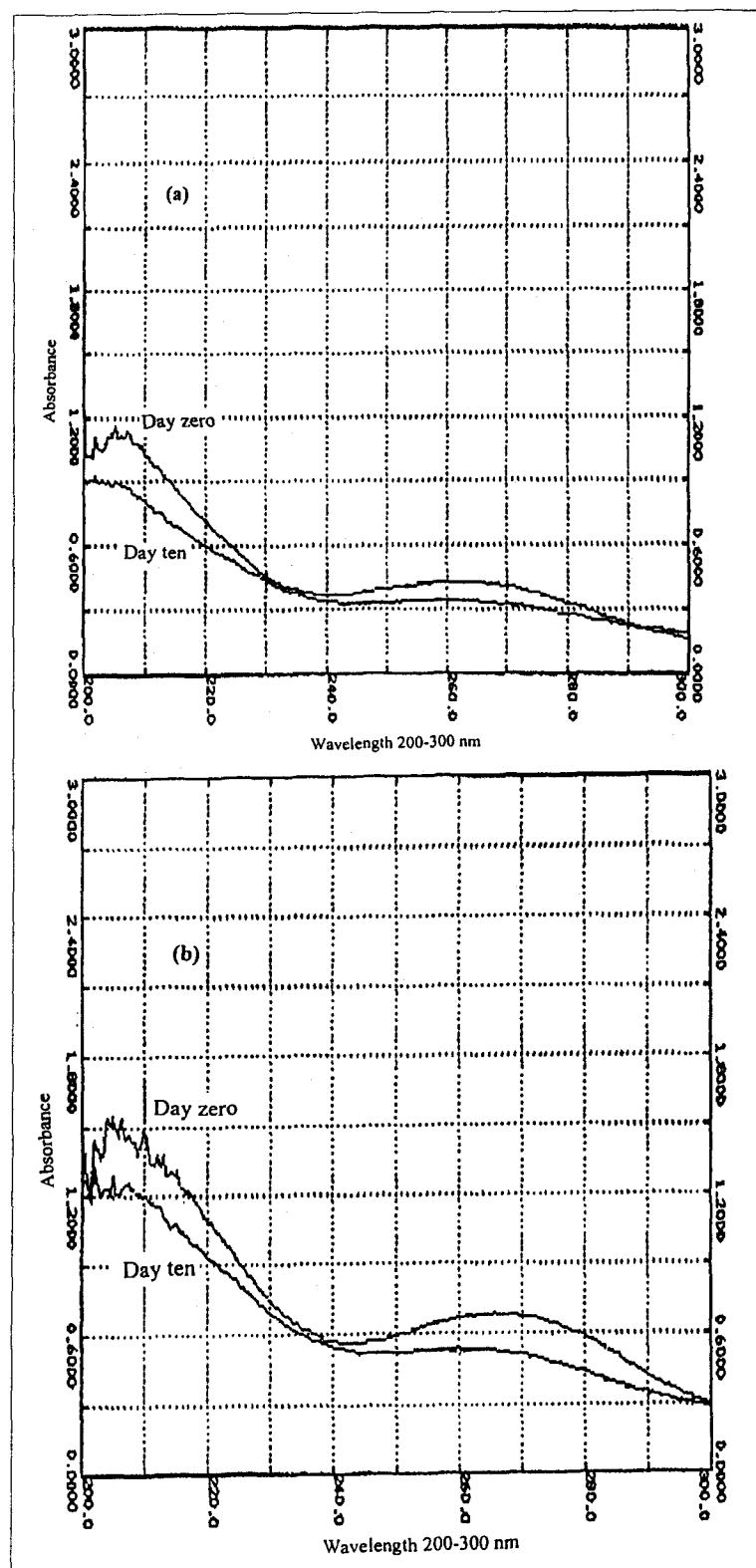


Figure 7. UV absorbance change of 10% (vol/vol) Dcs amended medium on day zero and after ten days of incubation with (a) *S. setonii* (b) *P. putida* suspend cell.

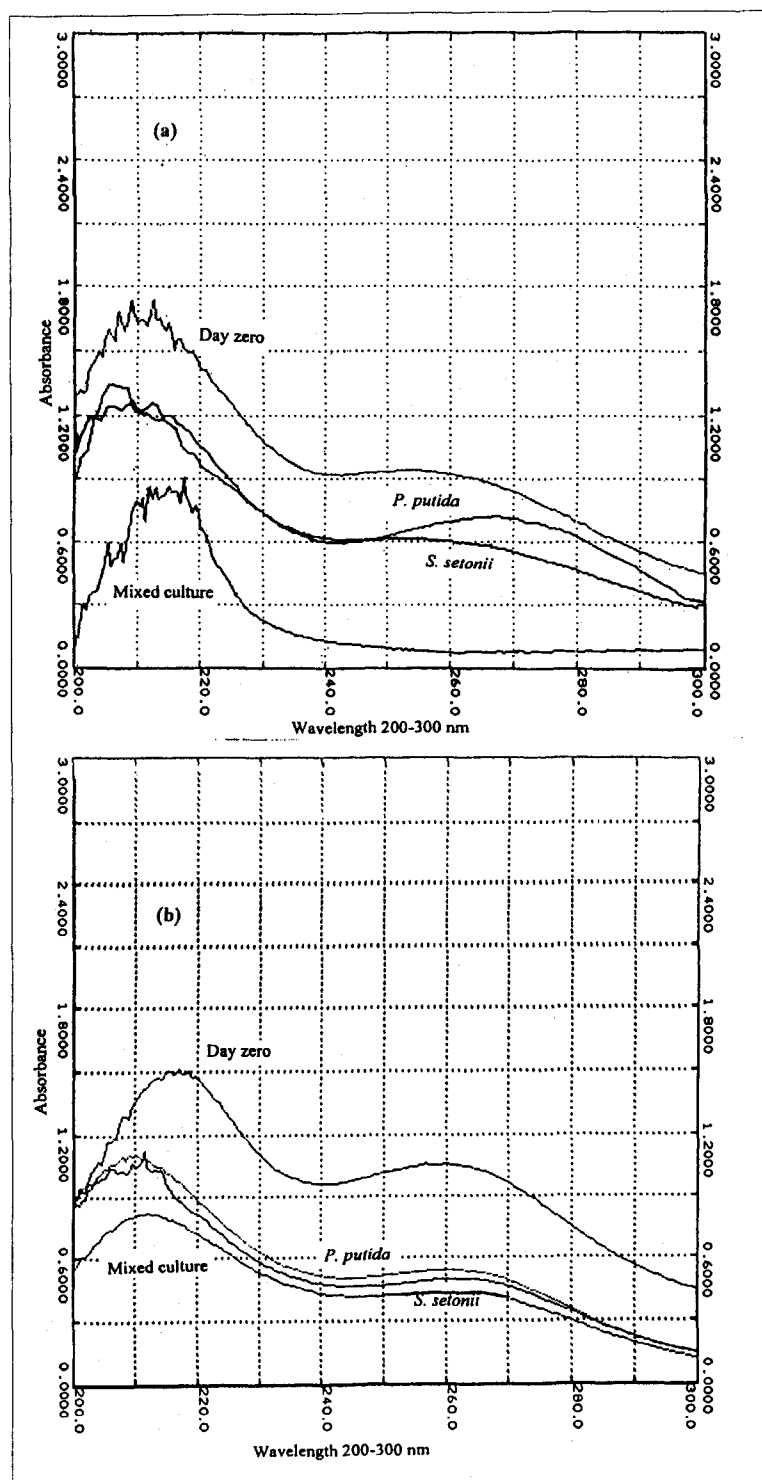


Figure 8. UV absorbance change of 25% (vol/vol) amended medium (a) Dcs and (b) Dst on day zero and after ten days of incubation with *P. putida*, *S. setonii* and mixed culture biofilm.

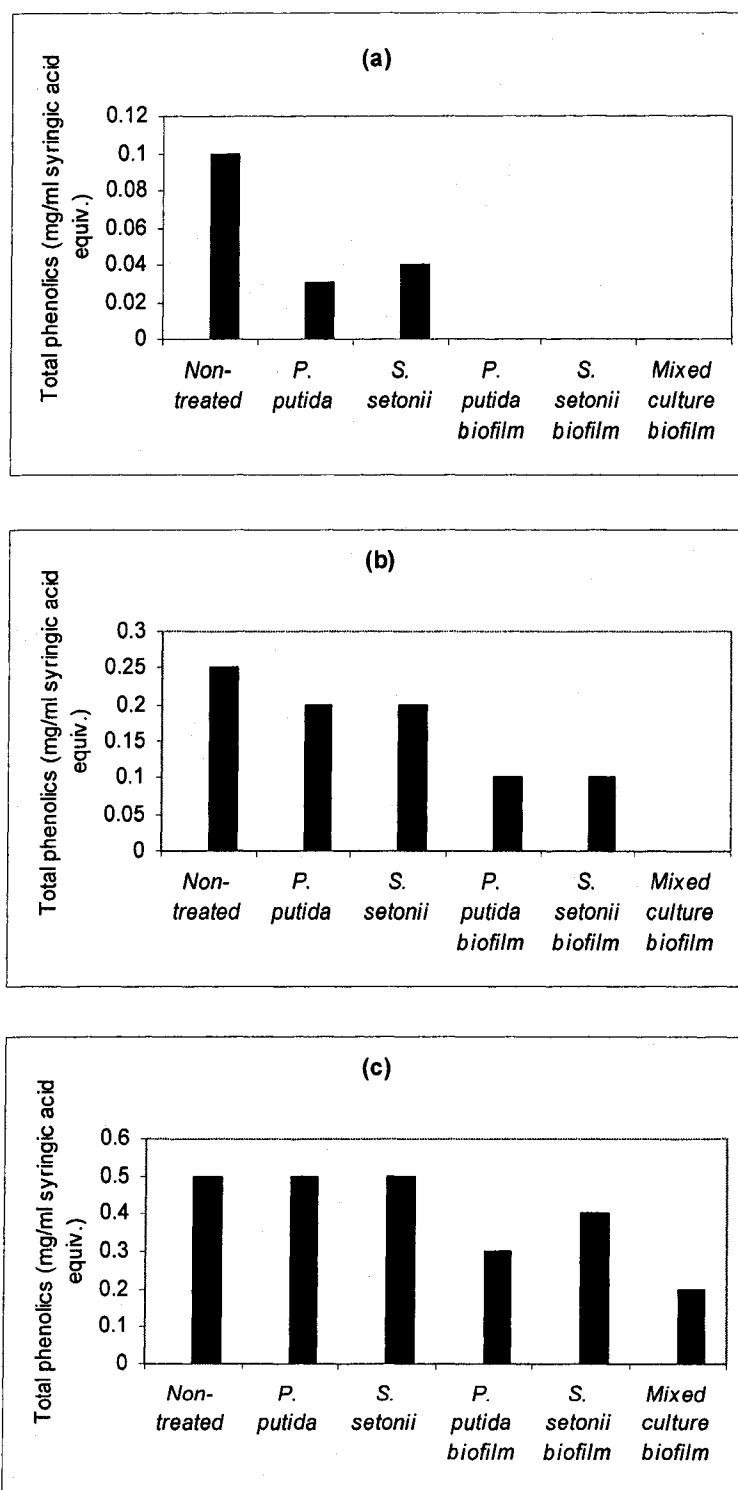
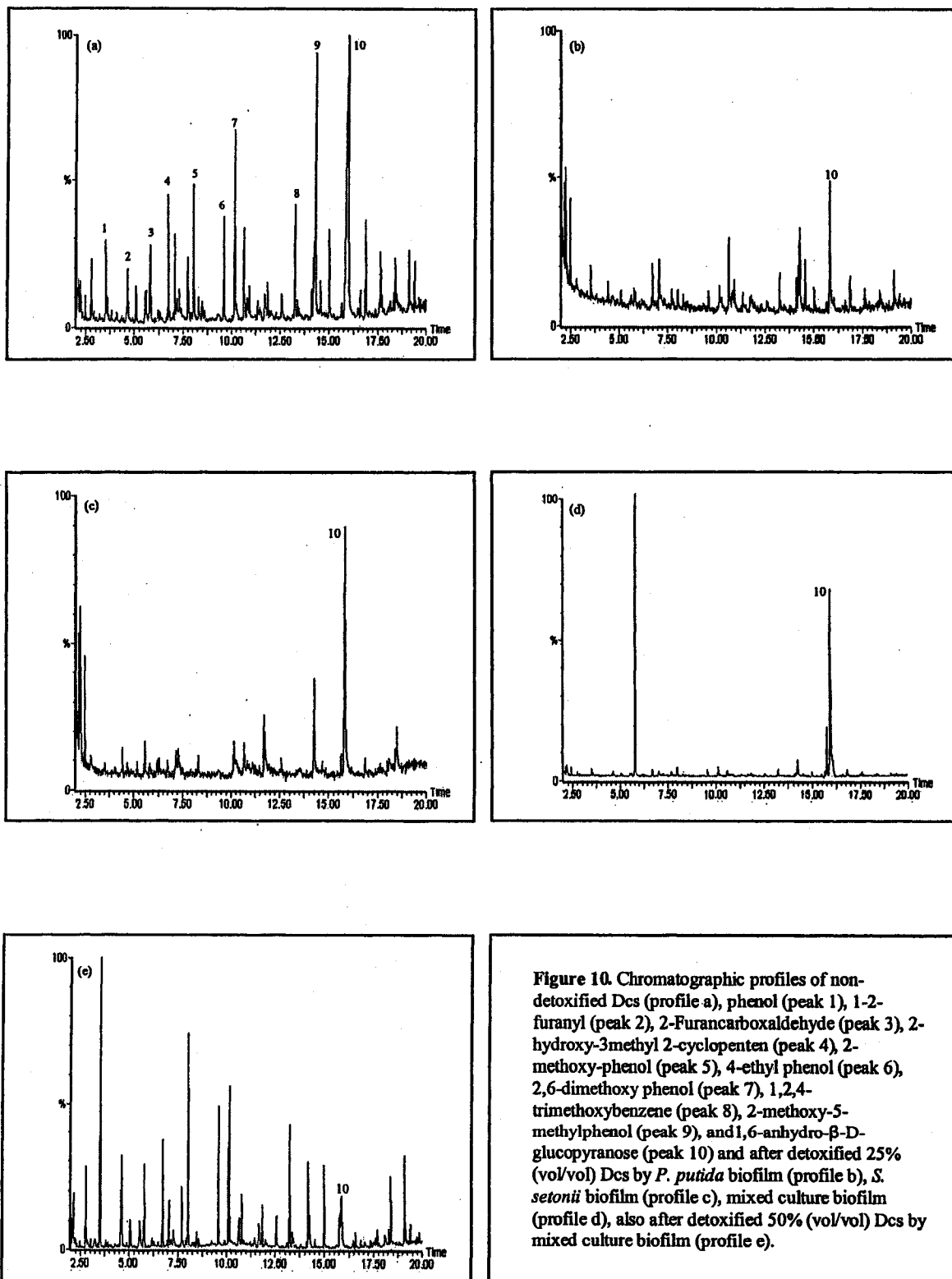


Figure 9. The reduction of total phenolics assay in (a) 10% (vol/vol) Dcs, (b) 25% (vol/vol) Dcs and (c) 50% (vol/vol) Dcs. After ten days of incubation.

GC-MS analysis

The analysis of non-detoxified Dcs and Dst samples showed many major peaks. The following compounds phenol, 1-2-furanyl, 2-Furancarboxaldehyde, 2-hydroxy-3methyl-cyclopenten, 2-methoxy-phenol, 4-ethyl phenol, 2,6-dimethoxy phenol, 1,2,4-trimethoxybenzene, 2-methoxy-5-methylphenol, and 1,6-anhydro- β -D-glucopyranose were identified in the non-detoxified Dcs. In the non-detoxified Dst, 2-Furancarboxaldehyde, 2(5H)-Furanone, 1,3-cyclopentanedione, 5-methyl-2-furancarboxaldehyde, 2-hydroxy-3methyl-cyclopenten, 3,6-dianhydro- β -D-glucopyranose, Furyl-hydroxymethyl ketone, 3,6-dianhydro-D-glucopyranose, 5-hydroxymethyl-2-Furancarboxaldehyde, and 1,6-anhydro- β -D-glucopyranose were identified.

The detoxified Dcs and Dst samples showed reduction in some peaks. The reduction was more noticeable in 10 and 25 % (v/v) Dcs and Dst (Figure 10, 11) compared to the reduction in 50 % (v/v) Dcs and Dst. Table 3 includes the reduction quantities of some compounds in Dcs and Dst detoxified samples.



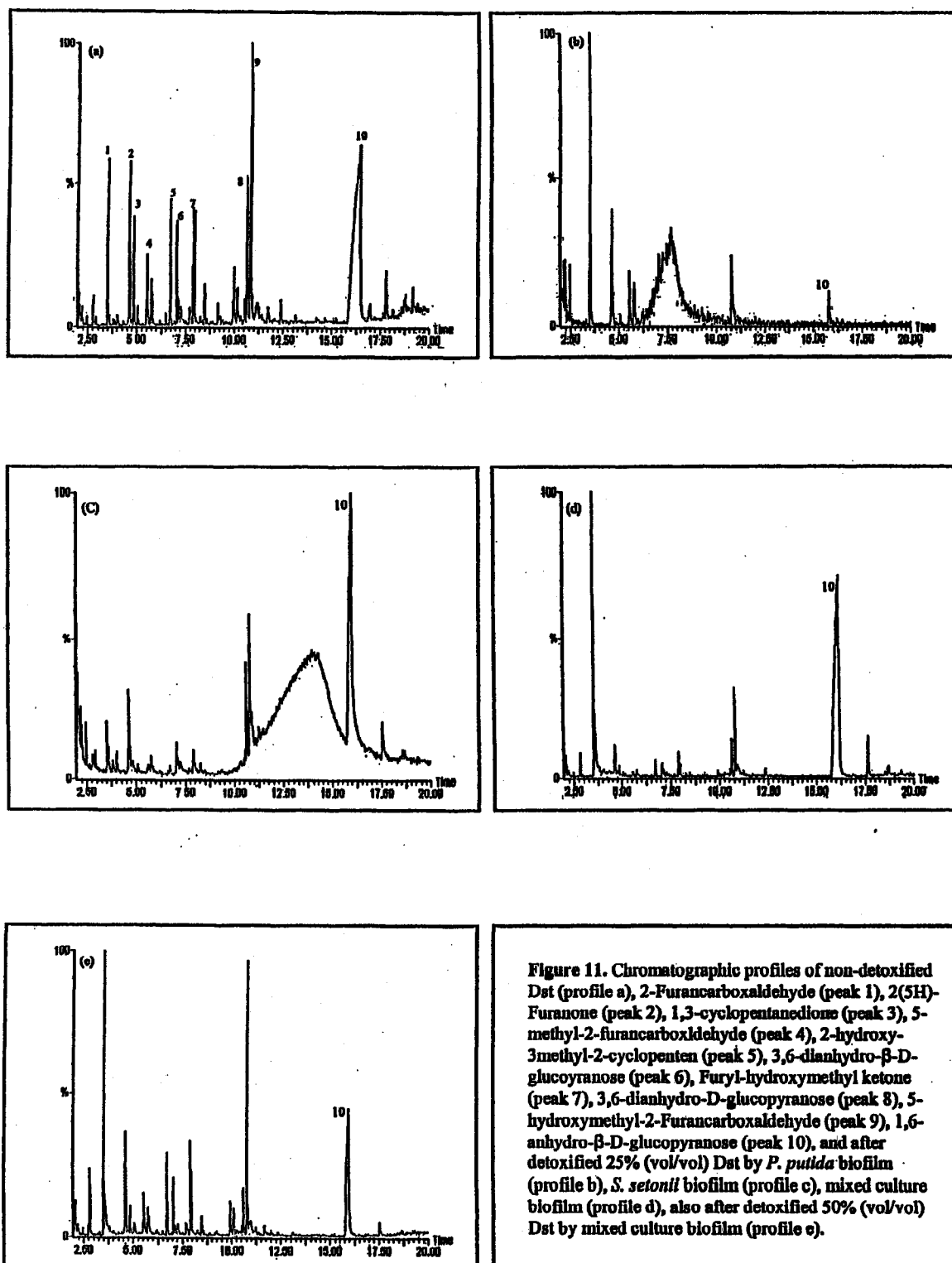


Table 4. The GC-MS quantitative analysis results of some compounds in Dcs and Dst before and after detoxification by *P. putida*, *S. setonii*, and mixed culture biofilms^a

Treatments	Compounds ^b in molar concentration					
	phenol	2-methoxy-5-methylphenol	2,6-dimethoxyphenol	1,2,4-trimethoxybenzene	levoglucosan	2(5H)furanone
Dcs ^c 25% (v/v) no-detoxified	6.0×10^{-4}	2.3×10^{-3}	2.6×10^{-3}	1.4×10^{-4}	0.12	ND ^d
Dcs 25% (v/v) detoxified by <i>P. putida</i> biofilm	5.3×10^{-5}	6.2×10^{-5}	2.0×10^{-4}	7.0×10^{-6}	0.11	ND
Dcs 25% (v/v) detoxified by <i>S. setonii</i> biofilm	3.8×10^{-5}	0.0	6.2×10^{-5}	0.0	0.11	ND
Dcs 25% (v/v) detoxified by mixed culture biofilm	1.8×10^{-5}	0.0	0.0	0.0	0.11	ND
Dcs 50% (v/v) no- detoxified	1.3×10^{-3}	4.7×10^{-3}	5.3×10^{-3}	2.8×10^{-4}	0.24	ND
Dcs 50% (v/v) detoxified by mixed culture biofilm	6.2×10^{-3}	7.6×10^{-4}	7.8×10^{-4}	3.3×10^{-5}	0.2	ND
Dst ^e 25% (v/v) no detoxified	ND	ND	ND	ND	0.24	1.1×10^{-2}
Dst 25% (v/v) detoxified by <i>P. putida</i> biofilm	ND	ND	ND	ND	0.24	3.2×10^{-3}
Dst 25% (v/v) detoxified by <i>S. setonii</i> biofilm	ND	ND	ND	ND	0.24	2.9×10^{-3}
Dst 25% (v/v) detoxified by mixed culture biofilm	ND	ND	ND	ND	0.22	2.1×10^{-3}
Dst 50% (v/v) no-detoxified	ND	ND	ND	ND	0.49	2.2×10^{-2}
Dst 50% (v/v) detoxified by mixed culture biofilm	ND	ND	ND	ND	0.46	6.2×10^{-3}

^a The quantitation analysis was determined according to pure standard for each compounds

^b The selected compounds for GC-MS quantitative analysis

^c Concentrations of non-detoxified and detoxified diluted corn stover

^d Compounds not detected in Dcs and Dst

^e Concentrations of non-detoxified and detoxified diluted corn starch

Bioassay

This assay showed that the detoxification of 10% (vol/vol) Dcs and Dst by mixed culture biofilm was more effective than the detoxification by *P. putida* and *S. setonii* suspended cell or biofilm. Therefore, when *L. casei* grew on detoxified 10% (vol/vol) Dcs by *P. putida*, and *S. setonii* suspended cell, and *P. putida*, *S. setonii*, and mixed culture biofilms produced 3.2, 5.3, 7.2, 8.3 and 17.7 g/l lactic acid, respectively. Similar results were found with the detoxified 25% (vol/vol) Dcs, the lactic acid was 1.8, 3.4, 5.1, 7.2, and 16.3 g/l with *P. putida*, and *S. setonii* suspended cell, and *P. putida*, *S. setonii*, and mixed culture biofilms (Table 3). The *L. casei* did not produced lactic acid with detoxified 50% (vol/vol) Dcs and Dst by *P. putida* and *S. setonii* suspended cell or *P. putida* and *S. setonii* biofilm. However with detoxified 50% (vol/vol) Dcs by mixed culture biofilm the *L. casei* produced 9.8 of g/l lactic acid. Like the detoxified 10 and 25% (vol/vol) Dcs the detoxified 10 and 25% (vol/vol) Dst was encouraging the *L. casei* to grew and produced lactic acid. The *L. casei* produced 17.9 and 13.4 g/l of lactic acid with the detoxified 10 and 25% (vol/vol) Dst by mixed culture which is higher then that produced from the detoxified 10 and 25% (vol/vol) Dst by *P. putida*, and *S. setonii* suspended cell, and *P. putida*, and *S. setonii* biofilms. Also, with detoxified 50% (vol/vol) Dst by mixed culture biofilm the *L. casei* grew and produced 6.8 g/l of lactic acid.

Discussion

In this study, the ability of two aerobic bacteria *P. putida* and *S. setonii* to detoxify diluted corn stover and diluted corn starch pyrolysis liquors produced by fast pyrolysis process was demonstrated. The goal was to develop a biological process that would permit

the biodegradation of pyrolysis liquors for industrial microbial fermentation. To our knowledge, this is the first report aimed to employ microorganisms to detoxify the toxic compounds in this effluent. Previous research employed activated carbon or organic solvents to extract the recalcitrant toxic compounds from pyrolysis liquors (32, 36).

Preliminary work with Dcs and Dst revealed high viscosity, low acidity and toxic compounds, which inhibit microbial growth. Analytical studies of various pyrolysis liquors identified a wide range of chemical compounds most of them are toxic to microbial growth (28, 36). The compounds found in the cellulose pyrolysate formed in the common range of pyrolysis temperatures, (500 to 650°C) are monosaccharide, anhydrosugars, carbonyl compounds, furans and lactones, pyrans, phenols, acids and acid esters and other compounds (28). The phenolic compounds in the pyrolysis liquors are derived from lignin in corn stover or from the chemical modification to corn starch which aimed to improve the functionality for practical application (28). However, in this study GC-MS analysis to non-detoxified corn starch pyrolysis liquor showed no phenolic compounds (Figure 10), but it has other toxic compounds such as furan, and furfurals derivatives (Figure 11). The dilution of the corn stover and the corn starch pyrolysis liquors was two fold, to remove the insoluble portion and to collect the supernatant (the water-soluble compounds), which is more biodegradable than the non-water-soluble compounds (45). The supernatant of diluted Dcs and Dst were neutralized with lime and filter sterilized, since heat sterilize hydrolyzes levoglucosan to glucose (36).

P. putida and *S. setonii* were selected based on their ability to metabolize variety of toxic compounds as a source of energy and carbon (38, 44) and their inability to metabolize levoglucosan. Both the flasks and the plates' cultures with Dcs or Dst and levoglucosan

(Table 1) confirmed *P. putida* and *S. setonii* ability to grow on Dcs and Dst. The liquid simple media consisted of yeast extract (Ardamine Z) with different concentration of Dcs or Dst with or without CaCO_3 was beneficial on industrial scale and recorded a good effect with the biodegradation. The *P. putida* and *S. setonii* suspended cell have the ability to oxidize different aromatic compounds (4, 8, 34, 38, 44) so they grew on the simple media with 10% (vol/vol) Dcs and Dst. The toxicity of Dst seems to be from the furfural derivatives (Figure 11) and growing the *P. putida* and *S. setonii* on 10% (vol/vol) Dst account for the detoxification of the furfural derivatives. The *S. setonii* only grew on 25% (vol/vol) Dcs and Dst, whereas the *P. putida* (Table 2) was not able to grow at 25% (vol/vol) Dcs and Dst, which accounted for the difference in their detoxification abilities. The differences in the detoxification degree between the individual strains capable of cometabolic degradation of compounds were found varying among strains (5). It has been noted that microbial degradation of compound mixtures is strongly impacted by other substituents in the mixture and the rate of consumption is also affected by the presence of these other compounds (38). But the mixture contains low concentrations such as 0.3 mM can be degraded by pure culture or mixed culture (3). The UV absorption spectra (Figure 7) and the bioassay (Table 3) confirmed the *P. putida* and *S. setonii* ability to biodegrade some toxic compounds in the Dcs and Dst. The phenol assay showed remaining of some phenols in 10, 25, and 50% (vol/vol) Dcs (Figure 9). The bioassay also showed that the suspended cell did not remove the toxic compounds from the 25 and 50% (vol/vol) Dcs, which inhibited the detoxification process.

Several studies mention that the biofilm is more resistant and degradable to toxic compounds (10, 50), but not all microorganisms can form biofilm. The plastic composite supports (PCS) stimulated the biofilm formation and enhance productivity of end products

(11, 14, 47). The *P. putida* biofilm was developed on the PCS using the exchange between high and low dilution rate. The *Pseudomonas* has been reported to form biofilm under different conditions (19, 48, 52). The *P. putida* was slow to form biofilm on PCS hence thick good biofilm was formed on day 11, whereas the *S. setonii* biofilm was defined on the PCS on day 2 with low dilution rates and the thick biofilm formed with increasing the dilution rates. In mixed culture, the *P. putida* and *S. setonii* grew and developed biofilm on the PCS. The mixed cultures PCS biofilm was allowed to develop based on the ability of the *S. setonii* to adhere to the PCS on day 2 and the *P. putida* on day 7, beside both *S. setonii* and *P. putida* grew well at pH 6.5 and 30°C. The advantage of developing mix culture is to degrade compound mixture through cooperative activity (3) and the degradation rate by mixed immobilized cell is higher than of suspended cell cultures (40).

In our study, the detoxification process showed that the *P. putida*, *S. setonii* and the mixed culture biofilms were more effective with low concentrations of (10 and 25%) Dcs and Dst. However, during the detoxification of 10% (vol/vol) Dcs and Dst, the DO₂ changed and NaOH were consumed rapidly from the media (Figure 6), whereas, the changes of DO₂ and NaOH during the detoxification of 25% (vol/vol) Dcs or Dst were variable during the ten days of incubation (Figure 6). Rapidly consuming of DO₂ during the first two days of incubation illustrates the ability of biofilm cells to remove some of inhibitor microbial compounds from the media. However, it seems that the biofilm cells were affected and biological activity decreased after day 2 until day 5. The DO₂ concentration remained high from day two to day five, which suggests the development of new biofilm culture. On day five, the DO₂ started to consume again from the media. Thus, the variation of DO₂ and NaOH consumption with 25% (vol/vol) Dcs and Dst suggests the possibility of developing

mutations due to the high concentration of the chemical compounds. The phenolic compounds assay showed completed reduction in 10% (vol/vol) Dcs (Figure 9) and the bioassay reported high amounts of lactic acid production (Table 3). Also, the GC-MS showed almost complete removal of detected compounds (Figure 10, 11).

The *P. putida*, *S. setonii* and mixed culture biofilms were also effective with the high concentrations of Dcs and Dst. The UV absorption change (Figure 8) showed the significance of the biofilm to reduce the toxic compounds in 25% (vol/vol) Dcs and Dst, especially the mixed culture biofilm. The phenolic compounds in 25% (vol/vol) Dcs was removed by mixed biofilm, while low amounts still remained after detoxification by *P. putida* and *S. setonii* biofilms (Figure 9). The GC-MS analysis also demonstrated disappearance or reduction of some peaks which account for removing or reducing the toxicity in Dcs and Dst (Figure 10, 11). These results were confirmed by increasing the cell density (A_{620}) and lactic acid production in the bioassay (Table 3). However, lactic acid produce from the detoxified 10% (vol/vol) Dcs and Dst was higher than that produced from detoxified 25% (vol/vol) Dcs and Dst. It is worth to mention that the 50% (vol/vol) Dcs and Dst were resistant to the detoxification by *S. setonii* and *P. putida* biofilm, but not to the mixed culture biofilm. Yet, the bioassay confirmed that the detoxification of Dcs was better than the Dst (9.8 and 6.8 g/l respectively). The small amount of lactic acid produced through detoxification of 50% (vol/vol) Dcs and Dst compared to the large amount produced from 10 and 25% (vol/vol) Dcs and Dst indicates a residual concentration of toxic compounds in the 50% (vol/vol). The overall results of the mixed culture biofilm illustrates cooperative metabolic activities between the two microorganisms, *S. setonii* and *P. putida* biofilms, which result in

detoxification of toxic compounds in both Dcs and Dst and which would be the process of choice.

In conclusion, the Dcs and Dst were demonstrated that mixture of chemicals in low concentrations can be detoxified using a PCS biofilms. The PCS mixed biofilm is an alternative approach to traditional mixed culture technique that employs undefined mixtures of environmental microorganism.

Acknowledgments

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Detoxification of Corn Stover and Corn Starch Pyrolysis Liquors by Ligninolytic Enzymes of *Phanerochaete chrysosporium*

A paper to be submitted to Applied and Environmental Microbiology

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Abstract

Phanerochaete chrysosporium (ATCC 24725) was able to grow and detoxify different concentration 10, 25, and 50% (vol/vol) of diluted corn stover (Dcs) and diluted corn starch (Dst) pyrolysis liquors in defined media. The GC-MS analysis illustrated reduction and change in some compounds also the total phenolic assay with Dcs samples demonstrated the reduction of the phenolics compounds. The bioassay confirmed removing toxic compounds of 10 and 25% (vol/vol) Dcs and Dst by the lignolytic enzymes, but not 50% Dcs and Dst. The addition of sodium azide or cycloheximide to *Ph. chrysosporium* culture media confirms the participation of lignolytic enzymes in the detoxification process. A concentrated enzyme preparation was able to detoxify the toxic compounds in corn stover and corn starch pyrolysis liquors as the fungal cultures.

Introduction

In the biosphere, plant biomass is our largest supply of stored carbon. One physical process beings evaluated to access this natural reservoir is pyrolysis which is a physical/chemical method for biomass saccharification (23). The dark brown mobile effluent,

generated by the pyrolysis process contains anhydrosugars, such as levoglucosan (1,6-anhydro β -D-glucopyranose), and a wide range of oxygenated chemicals compounds (11). Levoglucosan is a major product of hexosans pyrolysis, which can range from 38 to 58% depending on initial cellulose content (20, 26). Levoglucosan utilization by prokaryotes and eukaryotes is either directly as fermentable substrate or is indirectly through hydrolyzing it with mild acid to glucose (26, 28, 38). Unfortunately, the other compounds associated with the pyrolysis liquors inhibit microbial growth. The conventional methods of solvent extraction, chemical oxidation, and adsorbance on active carbons are employed to remove the inhibitor compounds from the pyrolysis liquor before using it as fermentable substrate (22, 28). These methods are costly and generate environmental disposal concerns. The use of biological treatment to transform or biodegrade these toxic compounds to non toxic compounds while leaving the levoglucosan seems promising (18).

Many microorganisms in particular white-rot fungi degrade a variety of recalcitrant molecules such as lignin, which is a polyphenylpropane molecule with β -aryl ether linkages (27). In *Phanerochaete chrysosporium* the lignin degradation system consists of peroxidases, H_2O_2 -producing enzymes, veratryl alcohol (3,4-dimethoxybenzyl alcohol), manganese, oxalate. However, *Ph. chrysosporium* is able to degrade chemicals via alternative pathways such as plasma membrane potential (4, 33). *Ph. chrysosporium* also has other enzymes or associated activities that may be important in remediation organic pollutants such as cellobiose dehydrogenase, a *trans*-membrane methyl transferase, *trans*-membrane redox potential, and laccase (7, 16). The lignin degradation system allows *Ph. chrysosporium* to biodegrade a wide range of compounds by oxidative and reduction mechanisms and to use highly reactive, nonspecific redox reactions, which increases the number of molecules that

can be effectively degraded via different alternative pathways (3, 7, 31). Since the ligninolytic system operate extracellular, it is ideal for use in the degradation a wide range of chemicals compounds that are either soluble or non-soluble in water. For example, *Ph. chrysosporium* can degraded lignin, munitions waste, pesticides, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, bleach plant effluent, synthetic dyes, synthetic polymers, and wood preservation (27). The corn stover and corn starch pyrolyzed at 400-600°C produces anhydrosugars such as levoglucosan, which can be fermented to organic acid or alcohol, and it produces a variety of toxic chemical compounds (6). Khiyami et al. (18) identified via gas chromatograph/ mass spectroscopy (GC-MS) analysis different phenolic and furfuryl derivatives compounds were present in diluted corn stover and diluted corn starch pyrolysis liquors these compounds inhibit microbial growth. Thus, *Ph. chrysosporium* ability to detoxify these pyrolysis liquors was evaluated.

In the present study, *Ph. chrysosporium* was cultured on defined medium with different concentrations of diluted corn stover or diluted corn starch pyrolysis liquors and also, concentrated extracellular ligninase was added to these liquors to evaluated detoxification. Since *Ph. chrysosporium* degrades organic pollutants via alternative pathways other than ligninolytic activity (7), the involvement of the peroxidases in the detoxification process was confirmed via the addition of sodium azide, which inhibited the oxidation of veratryl alcohol in the presence of the lignin peroxidase and H₂O₂ (35). Also, the importance of ligninases in pyrolysis liquor detoxification was determined via cycloheximide addition, which blocks the incorporation of leucine into *Ph. chrysosporium* protein synthesis which resulted in no extracellular ligninase production (17). The detoxification process was

determined via different parameters including measuring the extracellular proteins, ligninolytic activities, the reduction of total phenolic, bioassay tests, and GC-MS analysis.

Material and methods

Chemicals

Cycloheximide was obtained from ICN Biomedicals Inc. (Aurora, OH). Veratryl alcohol was obtained from Aldrich Chemical Company Inc. (Milwaukee, WI). The 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), hydrogen peroxide, syringic acid, polyethylene glycol compound and protein assay kit, 2-methoxy-5-methylphenol, 2,6-dimethoxy phenol, 1,2,4-trimethoxybenzene, 2(5H)furanone, and 1,6-anhydro- β -D-glucopyranose (levoglucosan) were obtained from Sigma Chemical Company (St. Louis, MO).

Microorganisms

Phanerochaete chrysosporium (ATCC 24725) and *Lactobacillus casei* subsp. *rhamosus* (ATCC 11443) strain were obtained from American Type Culture Collection (Manassas, VA). The *Ph. chrysosporium* strain was maintained on potato dextrose agar slants at 4°C. The *L. casei* was maintained as freeze-dried culture and as working culture by monthly transfers in MRS broth (Difco Laboratories, Detroit, MI) and stored at 4°C.

Pyrolysis liquors preparation

The pyrolysis process was carried out in a pilot-scale fluidized pyrolyzer at Iowa State University (6). The reactor consists of a 15-cm dia. bubbling fluidized bed operated at

400 – 600°C. It processes 2.5 – 5.0 kg/h of biomass to produce liter quantities of pyrolysate. Heat to drive pyrolysis is provided by burning pyrolytic gas or natural gas in an external burner and directing this hot gas through a heat exchange jacket surrounding the pyrolytic reactor. Recirculated pyrolytic gas serves as the fluidization agent with bubbling bed, thus avoiding addition of air to the reactor, which would reduce the yield of pyrolysate. The products of pyrolysis include gas (a flammable mixture consisting of hydrogen, carbon monoxide, carbon dioxide, and some non-condensable hydrocarbons), particulate matter (ash and char), and condensable vapors. Particulate matter was removed by insulated cyclones designed to keep the gas stream at high temperature. The gases and vapors then enter a series of four, water-cooled, shell-and- tube heat exchanges designed to condense water and organic vapors, which forms the pyrolysate used in the fermentation trials. In this research, the pyrolysates were prepared from corn starch and corn stover which is the residual after harvested. It has 50% (wt/wt) cellulose with some hemicelluloses, and lignin (18). Corn starch was purchased from local grocery store which could be subjected to a variety of chemical modifications aimed to improve functionality for food applications. The pyrolysates generated in this pyrolysis process were called corn stover pyrolysis liquor and corn starch pyrolysis liquor. Both generated significant yields of levoglucosan and monosaccharide.

Dilute pyrolysis liquors preparation

Dilute pyrolysis liquors were prepared as described by Khiyami et al. (18). Briefly corn stover or corn starch pyrolysis liquors were diluted with deionized water at 1: 2 ratio vigorously mixed then stored overnight in cold room at 4°C. The insoluble precipitates (IP) were removed by centrifugation at 10,000 x g for 15 min at 10°C. The supernatants were

neutralized with CaCO_3 then centrifuged at $15,000 \times g$ for 20 min at 10°C . The supernatant of diluted corn stover (Dcs) and diluted corn starch (Dst) pyrolysis liquors were filter sterilized with $0.45 \mu\text{m}$ filter to minimize any changes in chemical composition that might result from heat sterilization.

Inoculum preparation

Ph. chrysosporium was cultured on media containing 10.0 g/l of glucose, 10.0 g/l of malt extract, 2.0 g/l of peptone (Difco lab) 2.0 g/l of yeast extract (Ardamine Z; Sensient Flavor, Juneau, WI), 1.0 g/l of asparagine, 2.0 g/l of KH_2PO_4 , 1.0 g/l of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001 g/l of thiamin and 20 g/l of agar (Sigma Chemical Co) (34). The slants were incubated for five days at 39°C . Spore suspensions were prepared in sterile water followed by passage through sterile glass wool to remove mycelia. Finally, spores concentration was determined by measuring absorbance at 650 nm (34).

Pyrolysis liquors detoxification

The Dcs and Dst detoxification was carried out in 2-liter Erlenmeyer flasks. The flasks were capped with solid norprene stoppers with two holes containing norprene tubing which were attached to glass microfiber filter ($0.2 \mu\text{m}$ pore size and 50 mm Dia ; Whatman Inc. Colfton, NJ) to provide filter sterile air inlet and outlet (Figure 1). Each flask contained one-liter total volume of *Ph. chrysosporium* ligninase production media with 10, 25, or 50% (v/v) filter sterilized Dcs or Dst. The ligninase production media consist of 20.0 g/l of glucose, 0.22 g/l of ammonium tartrate, 2.0 g/l of KH_2PO_4 , 5.0 g/l of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g/l of $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 1.2 g/l of acetic acid, 0.4 g/l of NaOH, 11.7 ml/l of trace elements (34), 1.0 g/l of

Tween 80 (30), and 25 ml/l of spore suspension (0.83/ml at A_{650}). The flasks were purged with oxygen immediately after inoculation, tubing clamped then after day three the flasks purged daily with oxygen. The flasks incubated shaking at 120 rpm for 14 days at 39°C. On day three of incubation 3 mM of veratryl alcohol was added to the flasks cultures. On day four the flasks cultures were amended with 10, 25, or 50% (v/v) of filter sterilized Dcs or Dst. In order to determine the total reduction of phenolic compounds in Dcs samples, the above experiment under the same conditions was performed with 10 ml medium in 125 ml Erlenmeyer flasks. The total phenolic compounds were determined in triplicate flasks each two days for 14 days.

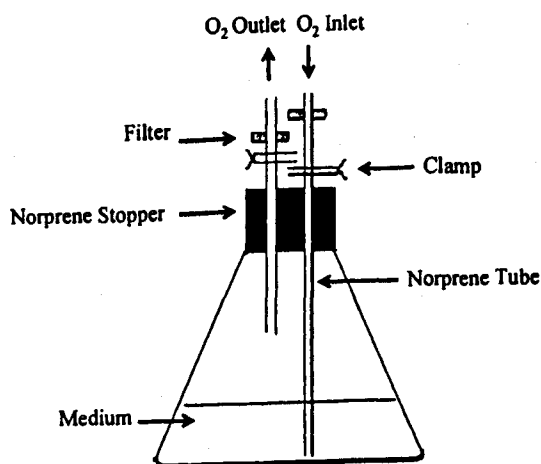


Figure 1. Set-up 2-liter Erlenmeyer flask agitated culture containing one liter medium with Dcs or Dst and oxygen purging tubing and filters.

Effect of inhibitors on ligninolytic system

In order to determine that detoxification of the Dcs and Dst was due to *Ph. chrysosporium* lignolytic enzymes, sodium azide and cycloheximide which inhibited the

enzyme production were added to *Ph. chrysosporium* culture medium. The experiment was carried out in triplicate with 10% (vol/vol) Dcs or Dst concentration. Sodium azide was added in 0 (control), 0.5, and 2.5 μM (35) to 10 ml of medium in a 125-ml Erlenmeyer flask. Cycloheximide was added in 0 (control) and 70 μg to 10 ml of medium in a 125-ml Erlenmeyer flask. These concentrations were added to culture on day two before ligninase production and day four after mycelium growing and ligninase production (13, 14). All experimental flasks were inoculated with 150 μl spore suspension (0.83/ml at A_{650}) and incubated for 6 days under the same conditions as describe previously in ligninase production and detoxification experiments.

Detoxification with concentrated enzymes

The ligninolytic enzymes were produced as described earlier in 2-liter Erlenmeyer flasks cultures, but without adding Dcs or Dst. When peroxidases (culture supernatant) reached the maximum activity on day 6 (19 and 31 U/L for LiP and MnP, respectively), the culture broth was centrifuged at 10,000 $\times g$ for 5 min at 4°C. The yellow supernatant, which contains ligninase activity, was added to dialysis tubing with molecular weight cut off 6000-8000 Daltons (Fisher Scientific, Spectrum lab. Inc. Roncho Dominguez, CA), placed in a tray covered with polyethylene glycol 15,000-20,000 MW (Sigma Chemical Co.), and stored at 4°C over night. The concentrated enzymes activity (28 and 43 U/L for LiP and MnP, respectively) were evaluated in detoxification experiments in 10 ml replicates of three. The reaction mixture contained 10% (vol/vol) Dcs or Dst, 3.0 ml concentrated enzyme, 0.1 M sodium tartrate (pH 4.5), 0.1 mM VA and 0.1 mM MnSO_4 . The reaction was initiated by addition of 0.1 mM H_2O_2 on four interval time 0, 15, 30 and 45 min. The control tube was

performed without H_2O_2 . The reactants were allowed to stir for 60 min and between the interval time samples were withdraw and later assayed for total phenolic compounds reduction. Finally, after 60 min all tubes were performed for bioassay.

Samples analysis

Lignin peroxidase (LiP)

The level of LiP activity was determined using veratryl alcohol assay (34) which contained 200 μl culture supernatant, 2 mM veratryl alcohol and 0.4 mM H_2O_2 in 50 mM sodium tartrate buffer, (pH 2.5). Oxidation of veratryl alcohol was measured as the increase in absorbance at 310 nm ($\epsilon = 9,300 \text{ M}^{-1} \text{ cm}^{-1}$). The reaction was monitored for 300 sec at 37°C using a DU 640 UV/Vis Beckman spectrophotometer equipped with kinetics Soft-Pac No. 517033 for measuring enzyme activity (Beckman Instruments, Inc. Fullerton, CA). One unite of LiP activity was defined as 1 μmol of veratryl alcohol oxidized in 1min under defined conditions, and activities were reported as U/l.

Manganese peroxidase (MnP)

The level of MnP activity was determined by monitoring the enzyme's oxidation of Mn^{2+} to Mn^{3+} (25). The assay mixture contained 200 μl culture supernatant, 0.1 M sodium tartrate (pH 5.0), 0.1 mM H_2O_2 , and 0.1 mM MnSO_4 . The product, Mn^{3+} , forms a transiently stable complex with tartaric acid, showing a characteristic absorbance at 238 nm ($\epsilon=6500 \text{ M}^{-1} \text{ cm}^{-1}$). Reactions were initiated by addition of H_2O_2 . The reaction was monitored for 300 sec at 37°C using a DU 640 UV/Vis Beckman spectrophotometer equipped with kinetics Soft-Pac No. 517033 for measuring enzyme activity. One unit of MnP activity was defined as 1

μmol of Mn^{2+} oxidize in 1 min under defined conditions, and activities were reported as U/l.

Laccase activity

The level of laccase was determined using 2,2'-azino-*bis*(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) as substrate (19). The reaction mixture contained 200 μl culture supernatant and 2 mM in 0.1 M sodium citrate buffer (pH 3.0). Oxidation of ABTS was monitored by measuring the absorbance increase at 420 nm ($\epsilon=36,000 \text{ M}^{-1} \text{ cm}^{-1}$). The reaction was monitored for 300 sec at 37°C using a DU 640 UV/Vis Beckman spectrophotometer equipped with kinetics Soft-Pac No. 517033 for measuring enzyme activity. One unit of laccase activity was defined as 1 μmol of ABTS oxidize in 1 min under defined conditions, and activities were reported as U/l.

Protein determination

The concentration of soluble protein in each sample was determined by Lowery reagent modified method (21) with crystalline bovine serum albumin as a standard.

GC-MS analysis

The samples were prepared and analyzed as described in Khiyami et al. (18). Briefly, 25 ml from detoxified and non-detoxified Dcs and Dst samples were acidified to pH 4.0 with HCl and then extracted three times with ethyl acetate, three times with ethyl acetate/acetone 2:1 and three times with ethyl ether (30). The pooled organic phases were dehydrated with anhydrous sodium sulphate and the solvents were removed via rotator evaporation. The dried extract was resuspended in 25 ml ethyl acetate and filtered. The analysis was performed

using Agilent 6890 series GC system with Agilent autosampler 7683 series injector and equipped with micromass GCT mass spectrometer, and column of JW DB.5 MS (30 m by 0.25 mm by 0.25 μ m, Agilent). The sample injected as split ratio 1 to 100. Also, five compounds in Dcs samples including phenol, 2-methoxy-5-methylphenol, 2,6-dimethoxy phenol, 1,2,4-trimethoxybenzene, and 1,6-anhydro- β -D-glucopyranose (levoglucosan) and two compounds in Dst including 2(5H)furanone and 1,6-anhydro- β -D-glucopyranose (levoglucosan) were used as standard to performed quantitation analysis in detoxified and non-detoxified Dcs and Dst samples. The levoglucosan is partially dissolved in ethyl acetate. For levoglucosan GC-MS quantitative analysis, 25 ml of detoxified and non-detoxified Dcs and Dst were dehydrated and then the dried extract were resuspended in 25 ml methanol.

Total phenols

The phenolic compounds were extracted as described above for GC-MS analysis then the dried extract was resuspended in 50 mM phosphate buffer (pH 6.0) instead of ethyl acetate. The phenolic assay is based on the oxidation of phenolate ion where the ferric ions are reduced to the ferrous state which was detected by the formation of the Prussian blue complex ($\text{Fe}_4[\text{Fe}(\text{CN})_6]_3$) with a potassium ferricyanide-containing reagent (37). The reagents of the assay were ferric chloride reagent, which was prepared by mixing 0.1 M solution of ferric chloride (FeCl_3) in 0.1 M hydrochloric acid, and potassium ferricyanide reagent, which was 0.008 M $\text{K}_3\text{Fe}(\text{CN})_6$ in deionized water (37). The assay mixture contained 25 ml of deionized water, 250 μ l of sample, 3 ml of ferric chloride reagent and 3 ml of potassium ferricyanide reagent. The sample absorbance was measured at 720 nm. The phenolic contents in the samples were expressed as syringic acid equivalents ($10 \mu\text{g ml}^{-1}$

gives an optical density of 0.377 at 760 nm) (1).

Unfortunately, any substance capable of being oxidized by the reagents of the total phenolic assay will yield the reduced (colored) forms of the reagents and appear as a phenolic. For example, ascorbic acid is one commonly occurring non-phenolic compound capable of doing this (37). Similar interfering result has been found with Dst (data not shown) although no phenolic compounds exist. Therefore, the total phenols assay was performed only with Dcs samples.

Bioassay

All samples collected for bioassay were centrifuged at 10,000 x g for 20 min at 20°C and then filter sterilized with a 0.45 µm filter membrane. The bioassay was performed using *L. casei* (18). The assay was carried out in a final volume of 10 ml by mixing 2:1 (vol/vol) of detoxified Dcs or Dst and MRS media. The assay tubes were inoculated with 100 µl (0.5/ml at A₆₂₀) of *L. casei* cultures and incubated at 37°C as a static culture for 24 hr. The control was incubated under the same conditions with non-detoxified Dcs or Dst. The samples were analyzed for cell concentration by absorbance at 620 nm using a spectronic 20 and lactic acid production by using Hewlett Packard (San Fernando, CA) high-pressure liquid chromatograph (HPLC) 1100 equipped with water model 2410 refractive index detector, column heater, autosampler, and computer controller. Lactic acid was separated on a Bio-Rad Aminex HPX-87H column (300 x 7.8 mm) (Bio-Rad Chemical Division, Richmond, CA) with 0.12 N sulfuric acid as the mobile phase at a flow rate of 0.8 ml/min with a 20-µl injection volume and a 65°C column temperature (8).

Results

Detoxification of Dcs and Dst

In order to study the effect of ligninolytic enzymes of *Ph. chrysosporium* to detoxify Dcs and Dst, different concentrations 10, 25, and 50% (vol/vol) of Dcs and Dst were added to the culture broth on day four so as not to inhibit fungal growth and MnP production. The *Ph. chrysosporium* was able to continue growing at all three concentrations of Dcs and Dst as determined by dry biomass weight measurement. Growth was slowest with 50% (vol/vol) Dcs and Dst. The dry mycelium weights average (replicate of two) were 1.7, 1.6 and 1.0 g/l with 10, 25 and 50% (vol/vol) Dcs, respectively, 1.6, 1.8 and 0.8 g/l with 10, 25 and 50% (vol/vol) Dst, respectively, and 2.2 g/l with control flasks (no Dcs or Dst addition).

GC-MS analysis

The analysis of non-detoxified Dcs and Dst samples showed many major peaks as described previously (18). In the non-detoxified Dcs, phenol, 1-2-furanyl, 2-Furancarboxaldehyde, 2-hydroxy-3methyl -cyclopenten, 2-methoxy-phenol, 4-ethyl phenol, 2,6-dimethoxy phenol, 1,2,4-trimethoxybenzene, 2-methoxy-5-methylphenol, and 1,6-anhydro- β -D-glucopyranose were identified (18). In the non-detoxified Dst, 2-Furancarboxaldehyde, 2(5H)-Furanone, 1,3-cyclopentanedione, 5-methyl-2-furancarboxaldehyde, 2-hydroxy-3methyl-cyclopenten, 3,6-dianhydro- β -D-glucopyranose, Furyl-hydroxymethyl ketone, 3,6-dianhydro-D-glucopyranose, 5-hydroxymethyl-2-Furancarboxaldehyde, and 1,6-anhydro- β -D-glucopyranose were identified (18).

The detoxified Dcs and Dst samples showed reduction in some peaks. The reduction was more noticeable in 10 and 25 % (vol/vol) Dcs and Dst (Figure 2, 3) compared to the

reduction in 50 % (vol/vol) Dcs and Dst. Table 1 includes the reduction quantities of some compounds in Dcs and Dst detoxified samples.

Extracellular proteins

In 10, 25 and 50% (vol/vol) Dcs the extracellular proteins concentration reached a maximum at day four at 17, 12 and 15 mg/l, respectively, and decreased gradually to 1.1, 0.8 and 1.0 mg/l by day 14, respectively (Figure 4). When 10% (vol/vol) Dst was added to medium, the extracellular protein level reached a maximum on day four with 15 mg/l and remained constant till day seven when it decreased to 0.1 mg/l. With 25 and 50% (vol/vol) Dst, the extracellular protein level reached a maximum on day six and eight with 13 and 15 mg/l, respectively. These amounts of extracellular protein decreased gradually to reached 0.3 and 0.6 mg/l by day 14 (Figure 4).

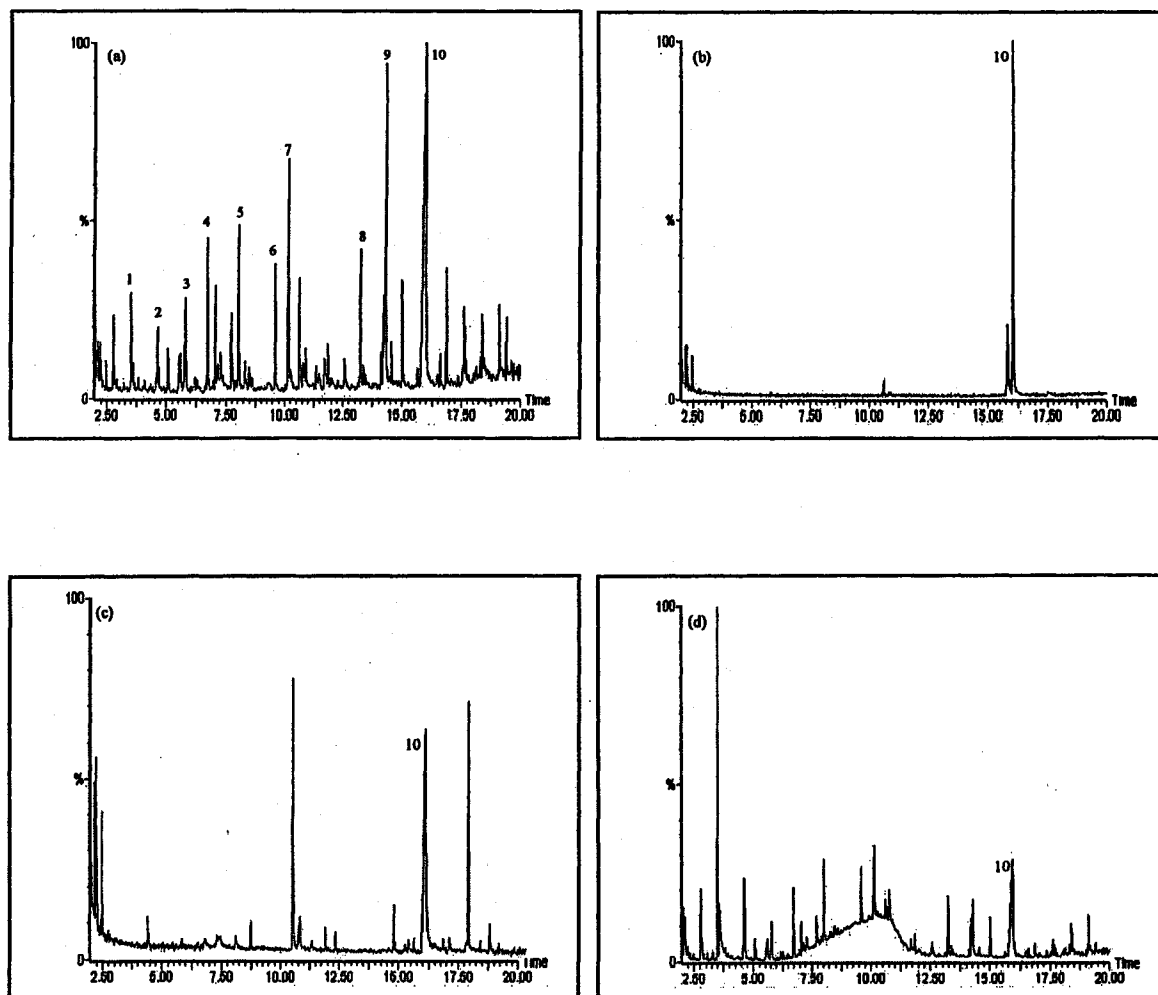


Figure 2. Chromatographic profiles of non-detoxified Dcs (profile a), phenol (peak 1), 1-2-furanyl (peak 2), 2-Furancarboxaldehyde (peak 3), 2-hydroxy-3methyl 2-cyclopenten (peak 4), 2-methoxy-phenol (peak 5), 4-ethyl phenol (peak 6), 2,6-dimethoxy phenol (peak 7), 1,2,4-trimethoxybenzene (peak 8), 2-methoxy-5-methylphenol (peak 9), and 1,6-anhydro- β -D-glucopyranose (peak 10) and after detoxified by *Ph. chrysosporium* 10% (vol/vol) Dcs (profile b), 25% (vol/vol) Dcs (profile c), and 50% (vol/vol) Dcs (profile d)

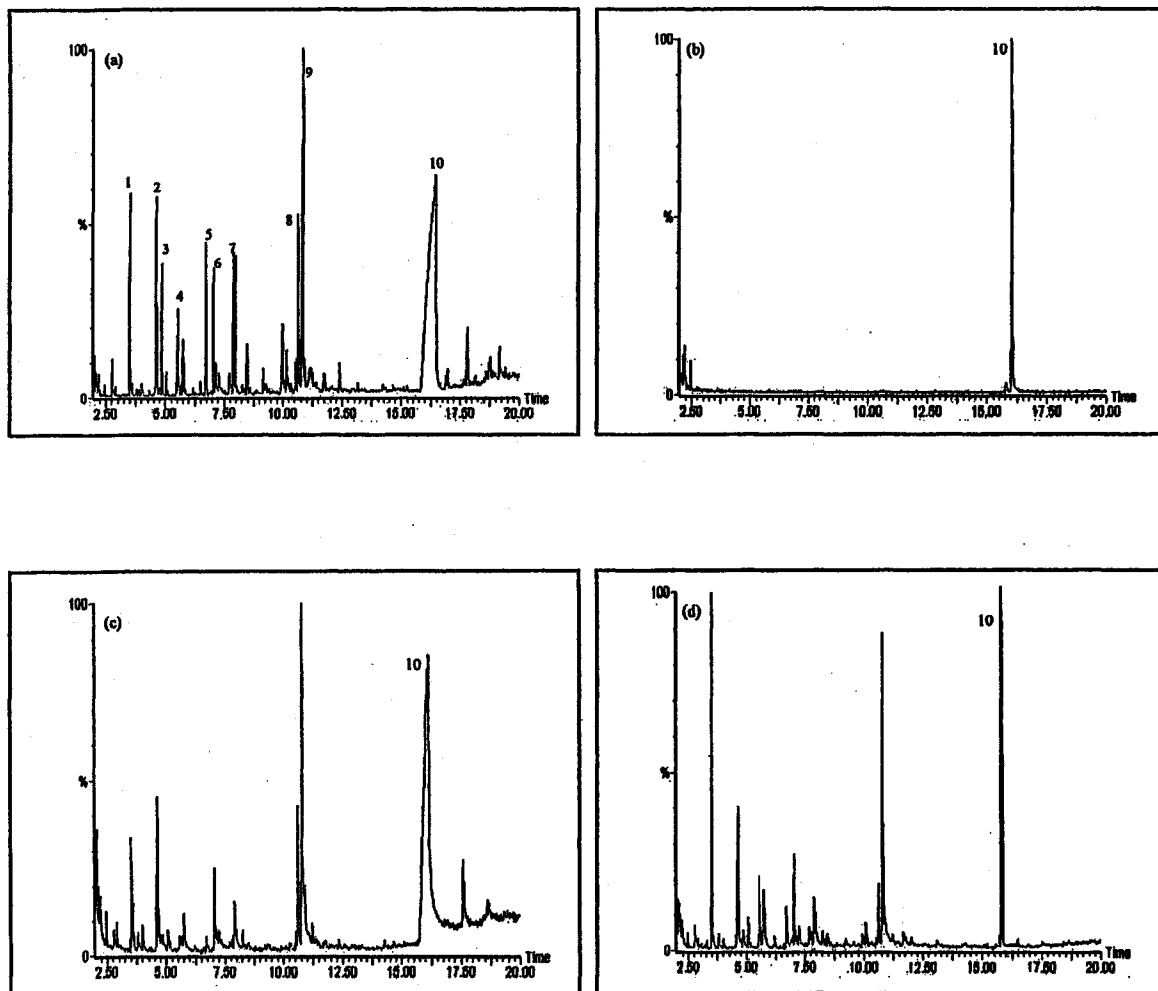


Figure 3. Chromatographic profiles of non-detoxified Dst (profile a), 2-Furancarboxaldehyde (peak 1), 2(5H)-Furanone (peak 2), 1,3-cyclopentanedione (peak 3), 5-methyl-2-furancarboxaldehyde (peak 4), 2-hydroxy-3methyl-2-cyclopenten (peak 5), 3,6-dianhydro- β -D-glucopyranose (peak 6), Furyl-hydroxymethyl ketone (peak 7), 3,6-dianhydro-D-glucopyranose (peak 8), 5-hydroxymethyl-2-Furancarboxaldehyde (peak 9) 1,6-anhydro- β -D-glucopyranose (peak 10), and after detoxified by *Ph. chrysosporium* 10% (vol/vol) Dst (profile b), 25% (vol/vol) Dst (profile c), and 50% (vol/vol) Dst (profile d)

Table 1. The GC-MS quantitation analysis results of some compounds in Dcs and Dst before and after oxidation by *Ph. chrysosporium*^a.

Treatments by <i>Ph. chrysosporium</i>	Compounds ^b in molar					
	phenol	2-methoxy-5-methylphenol	2,6-dimethoxy phenol	1,2,4-trimethoxy benzene	levoglucosan	2(5H)furanone
Dcs ^c 10 % (vol/vol) no-detoxified	2.5×10^{-4}	9.4×10^{-4}	1.1×10^{-3}	5.6×10^{-5}	0.05	ND ^d
Dcs 10 % (vol/vol) detoxified	0.0	0.0	0.0	0.0	NA ^e	ND
Dcs 25 % (vol/vol) no-detoxified	6.4×10^{-4}	2.3×10^{-3}	2.6×10^{-3}	1.4×10^{-4}	0.11	ND
Dcs 25 % (vol/vol) detoxified	0.0	0.0	0.0	0.0	0.11	ND
Dcs 50 % (vol/vol) no-detoxified	1.3×10^{-3}	4.7×10^{-3}	5.3×10^{-3}	2.8×10^{-4}	0.24	ND
Dcs 50 % (vol/vol) detoxified	1.9×10^{-2}	0.0	1.3×10^{-5}	0.0	0.23	ND
Dst ^f 10 % (vol/vol) no-detoxified	ND	ND	ND	ND	0.12	4.4×10^{-3}
Dst 10 % (vol/vol) detoxified	ND	ND	ND	ND	NA	0.0
Dst 25 % (vol/vol) no-detoxified	ND	ND	ND	ND	0.24	1.1×10^{-2}
Dst 25 % (vol/vol) detoxified	ND	ND	ND	ND	0.22	6.2×10^{-3}
Dst 50 % (vol/vol) no-detoxified	ND	ND	ND	ND	0.4	2.2×10^{-2}
Dst 50 % (vol/vol) detoxified	ND	ND	ND	ND	0.4	7.9×10^{-3}

^a The quantitation analysis was determined according to pure stander for each compounds (18)

^b The selected compounds for GC-MS quantitative analysis

^c Concentrations of diluted corn stover

^d Compounds not detected in Dcs and Dst

^e Compound not assay

^f Concentrations of diluted corn starch

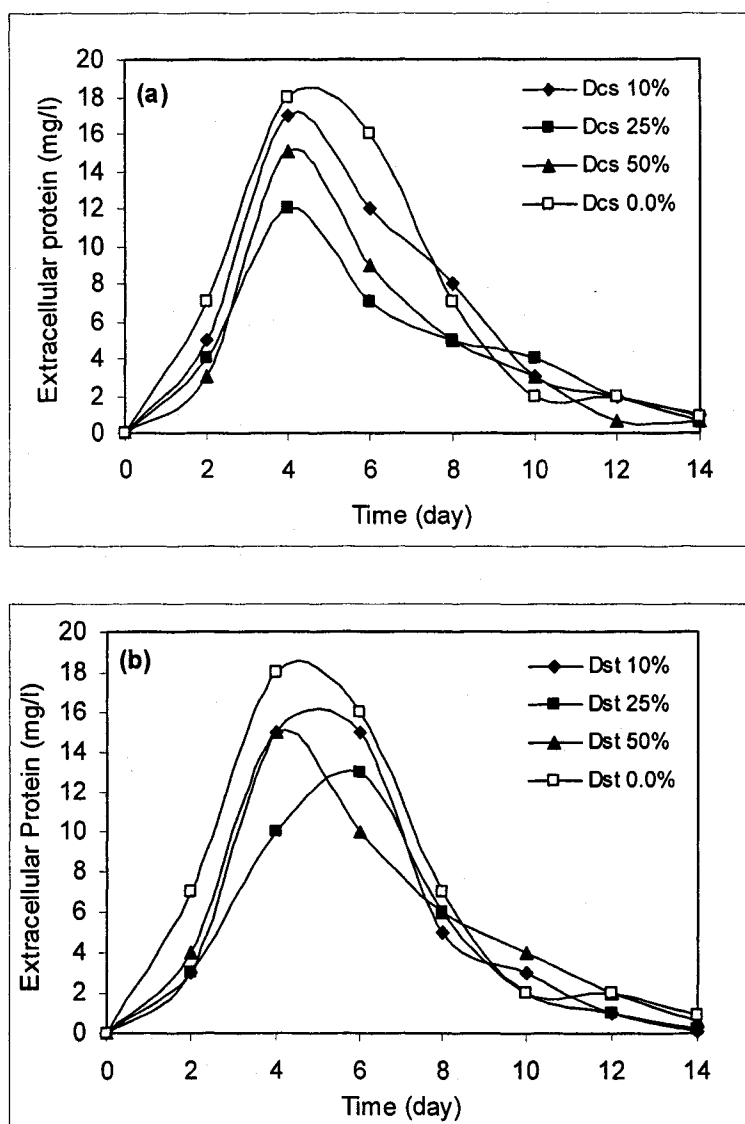


Figure 4. Extracellular proteins produced during the detoxification of 10, 25 and 50 and 0.0% (vol/vol) (a) Dcs and (b) Dst which were added on day four.

Ligninolytic activity during the detoxification

The activity of ligninolytic enzymes of *Ph. chrysosporium* in the shake flask culture media with different concentration of Dcs and Dst were monitored over 14 days. Laccase was not detected. In culture media with 10, 25 and 50% (vol/vol) Dcs or Dst, the MnP activity

was first detected on day two and was detected until day 14 (Figure 5). The MnP reached a peak on day six of a medium containing 10, 25 or 50% (vol/vol) Dcs. Similar result was found with 10% (vol/vol) Dst, whereas MnP reached peak on day four with 25% (vol/vol) Dst (Figure 5).

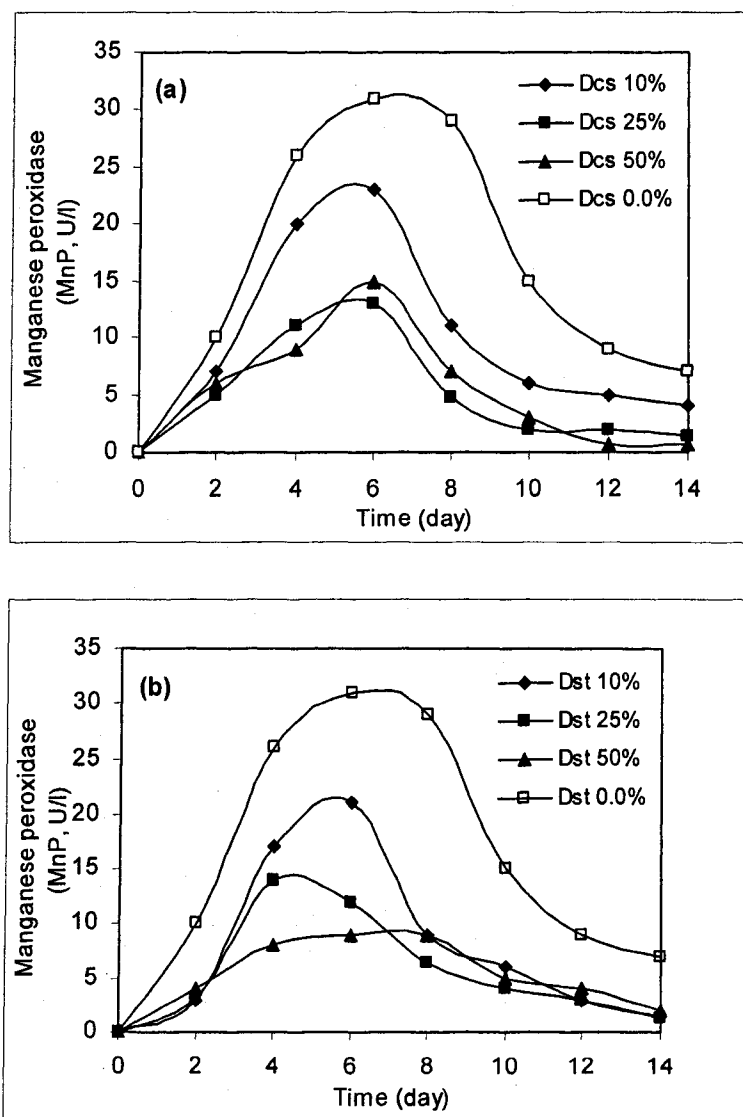


Figure 5. Manganese peroxidase produced by *Ph. chrysosporium* during the detoxification 10, 25, 50 and 0.0% (vol/vol) (a) Dcs and (b) Dst, which were added on day four to cultures.

The maximum MnP was 23, 14 and 15 U/L in culture media with 10, 25 and 50% (vol/vol) Dcs, and 21, 14 and 10 U/L in culture media with 10, 25 and 50% (vol/vol) Dst, respectively. The levels of MnP were decreased in the cultures media with all concentrations of Dcs and Dst by the end of study. In control flasks (no Dcs or Dst), the MnP reached a peak on day six with 31 and 7 U/L on day 14 (Figure 5).

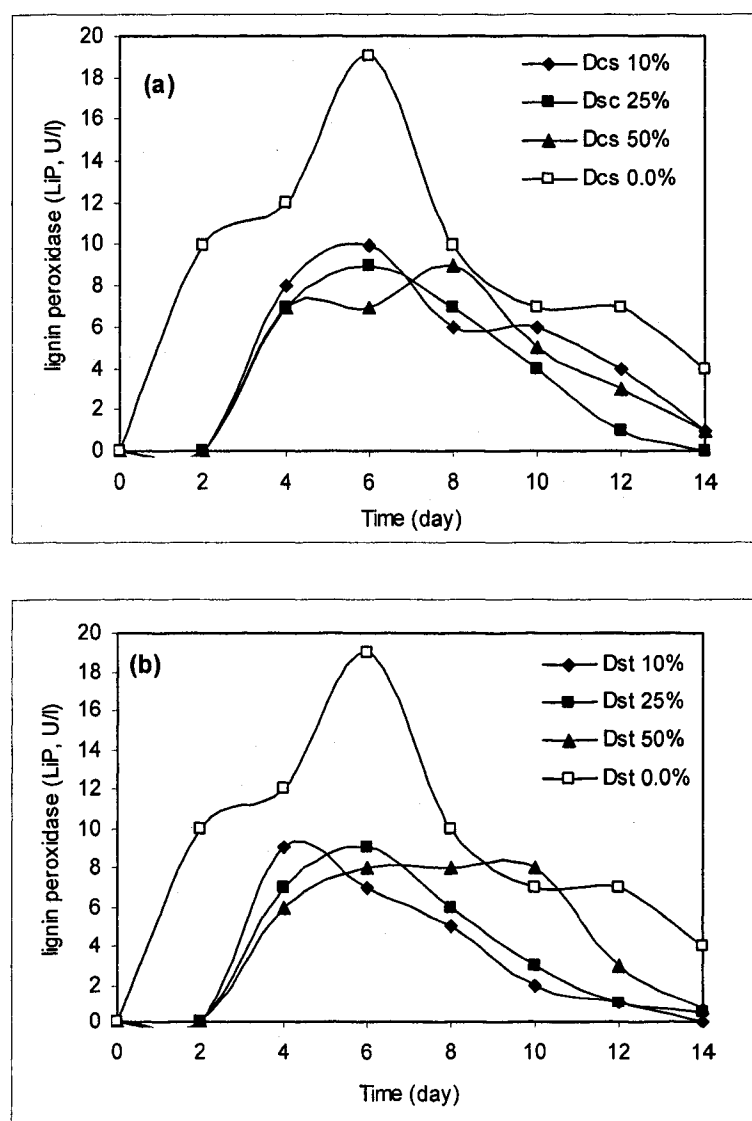


Figure 6. Lignin peroxidase produced by *Ph. chrysosporium* during the detoxification 10, 25, 50 and 0.0% (vo/vol) (a) Dcs and (b) Dst, which were added on day four to cultures .

The LiP activity was first detected on day four in culture media with 10, 25 and 50% Dcs or Dst (vol/vol) (Figure 6). The LiP activity reached a maximum on day six with 10 and 25% (vol/vol) Dcs and day eight with 50% (vol/vol) Dcs. The LiP reached a maximum on day four with 10% Dst (vol/vol), and on day six with 25 and 50% Dst (vol/vol). With 50% (vol/vol) Dst the LiP maintained 8 U/L for three consecutive days from day six to ten (Figure 6). The LiP was 10, 9 and 9 U/L in culture media with 10, 25, and 50% (vol/vol) Dcs, respectively, and 9, 9 and 8 U/L in culture media with 10, 25, and 50% (vol/vol) Dst, respectively. In control flasks (no Dcs or Dst addition) the LiP reached a peak on day six with 19 and 4 U/L on day 14. (Figure 6).

Phenolic compounds reduction

The *Ph. chrysosporium* was able to reduce most of the phenolic compounds in 10 and 25% (vol/vol) Dcs. The reduction of phenols content began to decrease gradually on day five (Figure 7). Over all, the *Ph. chrysosporium* almost completely biodegraded the phenolic compounds in 10 and 25% (vol/vol). However, in 50% (vol/vol) Dcs, the phenolic compounds concentration started to reduce on day four till day eight. After day eight, the phenolic compounds reduction was slowly and still existed in medium on day 14 (Figure 7). The concentrated ligninolytic enzyme reduced the total phenolics compounds in 10% (vol/vol) Dcs from 0.1 to 0.01 mg/ml after 60 minutes incubation (Figure 8).

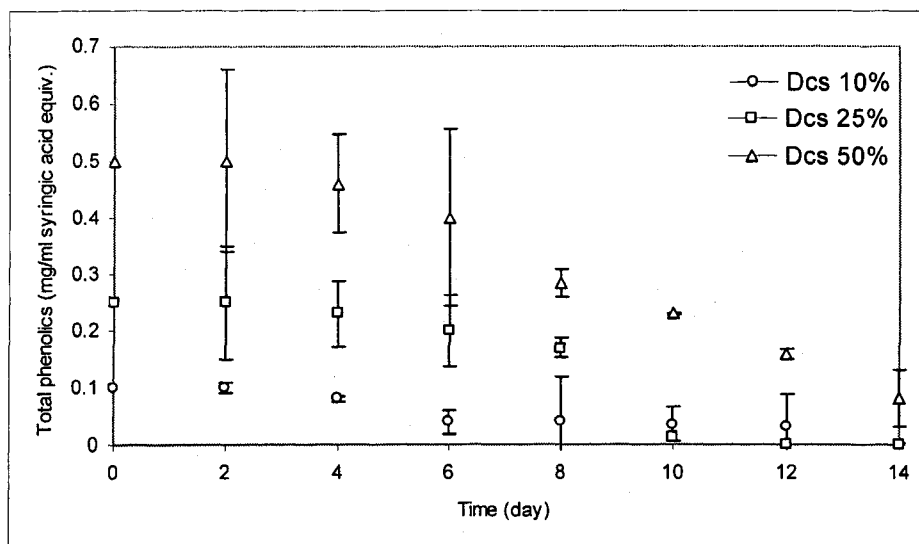


Figure 7. Total phenolic compounds reduction in 10, 25 and 50% (vol/vol) Dcs after 14 day of incubation with *Ph. chrysosporium*. Each reading represents the average of at least two replicate.

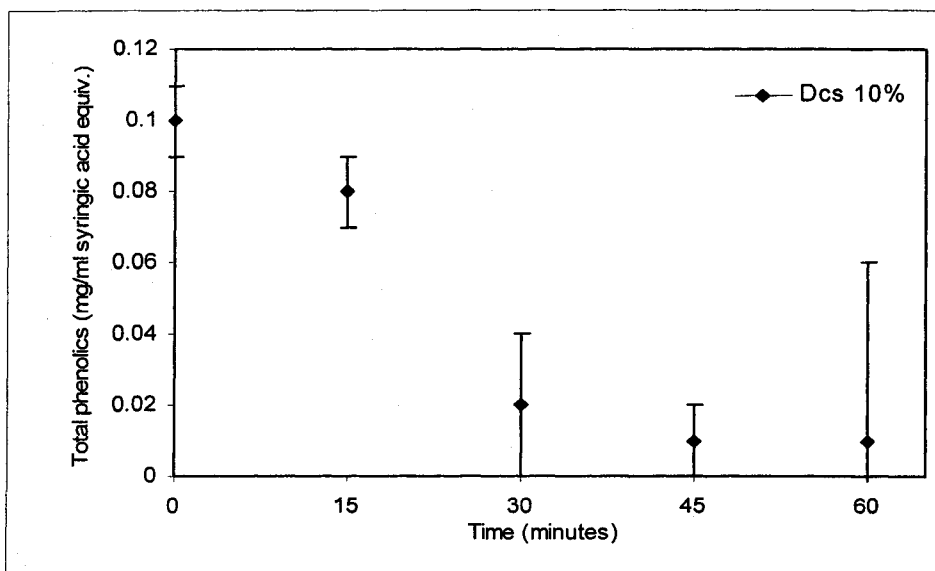


Figure 8. Phenolic compounds reduction in 10% (vol/vol) Dcs during 60 minutes of incubation with concentrated enzyme. Each point represents the average of three replicate. The initial LiP and MnP activity were 28 and 43 U/L, respectively. The reaction was initiated by addition of 0.1 mM H_2O_2 on four interval time 0, 15, 30 and 45 min.

Bioassay

The detoxified 10% (vol/vol) Dcs and Dst by the *Ph. chrysosporium* demonstrated the complete loss of Dcs and Dst toxicity, which was monitored by the growing *L. casei*. (Table 2). The A_{620} was 3.5 and 3.2, and the lactic acid productions were 10.4 and 9.5 g/l for 10% (vol/vol) detoxified Dcs and Dst, respectively. In 25% (vol/vol) detoxified Dcs and Dst the bioassay also demonstrated detoxification, but the A_{620} and lactic acid produced were significantly less than that produce with 10% (vol/vol) detoxified Dcs and Dst. The *L. casei* did not grow with 50% (vol/vol) detoxified Dcs or Dst.

Table 2. The ability of *L. casei* to grow on the 10, 25 and 50% (v/v) Dcs and Dst detoxified by *Ph. chrysosporium* and 10% (v/v) Dcs and Dst detoxified by concentrated enzyme (bioassay)^a

Treatments	Detoxified Dcs (vol/vol) ^b						Detoxified Dst (vol/vol) ^c					
	10%		25%		50%		10%		25%		50%	
	A_{620}	LA ^d g/l	A_{620}	LA g/l	A_{620}	LA g/l	A_{620}	LA g/l	A_{620}	LA g/l	A_{620}	LA g/l
Control ^e	- ^f	-	-	-	-	-	-	-	-	-	-	-
<i>Ph. chrysosporium</i> ^g	3.5	10.4	0.3	2.7	-	-	3.2	9.5	0.5	3.2	-	-
Concentrated enzyme ^h	1.7	5.3	NA ⁱ	NA	NA	NA	0.8	4.1	NA	NA	NA	NA

^a Bioassay was performed as described in (18). Each result represents the average of at least three replicate

^b Concentrations of detoxify (day 14 samples) diluted corn stover

^c Concentrations of detoxify (day 14 samples) diluted corn starch

^d Lactic acid produce due to growing *L. casei*

^e A mixture of MRS media and non detoxified samples (day zero samples), and for the concentrated enzyme assay, a mixture of MRS media and non detoxified samples (time zero samples) both inculcated with *L. casei*

^f No growth or lactic acid production

^g *Ph. chrysosporium* culture employing for detoxifying the Dcs and Dst

^h Concentrated enzyme of *Ph. chrysosporium* employing for detoxifying Dcs and Dst

ⁱ Not assayed

Inhibitors effect

To confirm detoxification of Dcs and Dst with *Ph. chrysosporium* ligninolytic enzyme, sodium azide and cycloheximide were added to the cultures flasks with 10% (vol/vol) Dcs or Dst. The Dcs and Dst cultures flasks amended with 0.5 and 2.5 μ M sodium azide on day two produced a small amount of mycelium, no ligninolytic enzyme (LiP), and negative bioassay (Table 3, 4). Whereas, Dcs and Dst culture flasks amended with 0.5 and 2.5 μ M sodium azide on day four produced more of mycelia, there was no LiP activity and *L. casei* was unable to grow in this detoxified liquors negative bioassay (Table 3, 4). Addition of 70 mg cycloheximide to cultures flasks with 10% (vol/vol) Dcs or Dst on day two produced a small amount of mycelium and no LiP was detected. Whereas, 70 mg cycloheximide addition to cultures flasks at day four produce more mycelia, higher LiP activity and the toxicity of 10% (vol/vol) Dcs and Dst was removed (Table 3, 4).

Table 3. Effect of sodium azide and cycloheximide on ligninolytic activity (LiP) during detoxification of 10 % (v/v) Dcs or Dst^a.

Treatments			Dcs ^b		Dst ^c	
	Con. ^d	Day ^e	LiP U/l ^f	Dry weight g/l	LiP U/l	Dry weight g/l
Sodium azide	0.0 μ M	2	8.0	1.3	7.0	1.0
	0.5 μ M		ND ^g	0.02	ND	0.02
	2.5 μ M		ND	0.01	ND	0.01
	0.5 μ M	4	ND	0.6	ND	0.7
	2.5 μ M		ND	0.3	ND	0.3
Cycloheximide	0.0 μ M	2	7.0	1.1	7.0	1.2
	70 μ M		ND	0.01	ND	0.01
	70 μ M	4	5.0	0.5	3.0	0.4

^a The experiment was ran for 6 days and each result represent the average of at least three replicate

^b 10% (vol/vol) diluted corn stove added to culture on day 4

^c 10% (vol/vol) diluted corn starch added to culture on day 4

^d Concentration of sodium azide and cycloheximide added to *Ph. chrysosporium* culture

^e Day of sodium azide and cycloheximide addition

^f LiP activity determined on day 6

^g Not detected

Table 4. The ability of *L. casei* to grow on the 10% (vol/vol) Dcs or Dst detoxified by *Ph. chrysosporium* in presence of sodium azide and cycloheximide (bioassay)^a.

Treatment		Detoxified Dcs ^b		Detoxified Dst ^c	
		A ₂₆₀	LA ^d g/l	A ₂₆₀	LA g/l
Sodium azide	0.0μM ^e	3.8	9.5	3.0	10.0
	0.5μM	- ^f	-	-	-
	2.5μM	-	-	-	-
	0.5μM ^g	-	-	-	-
	2.5μM	-	-	-	-
Cycloheximide	0.0μM ^h	3.3	8.3	1.5	6.2
	70μM	-	-	-	-
	70μM ⁱ	0.1	2.1	0.2	2.3

^a Bioassay was performed as described in (18). Each result represents the average of at least three replicate

^b Detoxify diluted corn stover

^c Detoxify diluted corn starch

^d Lactic acid produce due to growing *L. casei*

^e Concentrations of sodium azide added to *Ph. chrysosporium* culture on day 2

^f No growth or lactic acid production

^g Concentrations of sodium azide added to *Ph. chrysosporium* culture on day 4

^h Concentrations of cycloheximide added to *Ph. chrysosporium* culture on day 2

ⁱ Concentrations of cycloheximide added to *Ph. chrysosporium* culture on day 4

Discussion

This study demonstrates the feasibility of employing *Ph. chrysosporium* to remove toxic compounds from Dcs and Dst pyrolysis liquors instead of the conventional physical/chemical methods (22, 28). Most biodegradation studies using *Ph. chrysosporium* have been performed with fungal cultures (9, 16, 27, 36). These studies suggested that enzymes involved in detoxification are ligninase, which are also involved in xenobiotic degradation. The *Ph. chrysosporium* ligninase enzymes degrade several organo-pollutants

similar to lignin because it is non stereoselective and non specific (1, 5, 19, 27). The ligninases biodegradations of aromatic compounds depend on their structure and the oxidation rates, which increased in presence of methoxy and hydroxyl groups (18, 39). These enzymes affectively removed the toxic compounds in Dcs and Dst, but our ability to distinguish the specific compounds degraded was limited (Figure 2, 3). The reduction of toxic compounds as determined by GC-MS (Figure 2, 3) and the bioassay (Table 2, 4) were considered good parameters to elucidate the removal of toxic compounds in Dcs and Dst. Conformation of the phenolic compounds reduction in Dcs was also obtained with total phenolics compounds assays (Figure 7, 8). In 10, 25 and 50% (vol/vol) Dcs and Dst detoxification experiments, the *Ph. chrysosporium* produced 8 to 10 U/L of LiP and 10 to 23 U/L of MnP. The ligninolytic enzyme system of *Ph. chrysosporium* cultures flasks catalyzed the oxidation of toxic compounds as did ligninolytic enzyme concentrated with 10% (vol/vol) Dcs and Dst. These results also indicated that the ligninolytic enzymes (LiP and MnP) were produced in sufficient amounts during fungal growth to catalyze the oxidation of toxic compounds (Figure 5, 6). In 25% (vol/vol) detoxified Dcs and Dst the toxic compounds were also removed (Figure 2, 3), but the lactic acid production from the bioassay was low compared to 10% (vol/vol) detoxified Dcs and Dst bioassay (Table 2). In 50% (vol/vol) Dcs and Dst the incomplete removal of toxic compounds was confirmed by the bioassay with no detectable growth or lactic acid production, also the GC-MS analysis showed no change in the detoxified sample (Figure 2). To complete the detoxification in 25 and 50% (vol/vol) Dcs and Dst, increasing the period of incubation might be helped to remove the rest of the toxic compounds, since the transformations by *Ph. chrysosporium* cultures are slow (2). However, in bioremediation the long period of incubation is not desired thus concentrated enzyme

carried out the detoxification in hours, whereas fungus culture carried it out in weeks. The direct oxidation of chemical compounds by purified ligninase has been reported which seems to implicate the ligninolytic system directly in the detoxification of xenobiotics (15, 30, 36). In our result, the phenolic compounds in 10% (vol/vol) Dcs were removed within 60 min with concentrated enzyme (Figure 8), which was confirmed by detoxification in bioassay (Table 2).

Beside the ability of lignolytic enzymes degradation there is also evidence to suggest that white rot fungi possess other mechanism of xenobiotics degradation that are not reliant on the ligninolytic system. *Ph. chrysosporium* was observed to degrade the polycyclic aromatic hydrocarbon under ligninolytic as well as non-ligninolytic conditions, suggesting that the potential of *Ph. chrysosporium* for degradation of certain environmental pollutants is not limited to nutrient starvation conditions (10). So, it has been postulated that a plasma membrane potential might be involved in the bioconversion of various xenobiotics (4, 33). For example, several redox dyes have been shown to be possibly reduced via such a mechanism, and trinitrotoluene (TNT) may also be reduced by this method. It has been suggested that *Ph. chrysosporium* maintains the pH of its environment at about 4.5 via proton pumping and that bioconversion of chemicals may be linked to this proton gradient (4, 33). Therefore, the addition of sodium azide to the detoxify *Ph. chrysosporium* media on day two and four confirmed the ligninases role. Our result showed that the addition of sodium azide on day two and four of shake flask culture incubation inhibited LiP activity (Table 3) and inhibited toxic compounds reduction (Table 4). Sodium azide is a potent inhibitor of LiP activity. Inhibition of veratryl alcohol oxidase activity by sodium azide indicated that inhibition was due to reaction of sodium azide with activated enzyme, compound I, rather than binding of

sodium azide to the native enzyme (35). It is also thought to affect the plasma membrane redox system of the fungus (32).

Cycloheximide inhibits protein synthesis. It blocks leucine incorporation into proteins (17). Thus, the addition of the cycloheximide to the *Ph. chrysosporium* culture on day two or earlier will prevent ligninolytic enzyme production (17) and Dcs and Dst demonstrated toxicity in bioassay (Table 4). Both LiP and MnP are produced in idiophasic and cycloheximide addition would stop their production (13). Thus, when the cycloheximide was added on day four after the enzyme production excretion toxic compounds were removed from 10% (vol/vol) Dcs and Dst (Table 3, 4). Dosoretz et al. (12) demonstrated that addition of cycloheximide on day six to *Ph. chrysosporium* cultures resulted in no change of LiP activity.

In conclusion, *Ph. chrysosporium* was able to biodegraded the toxic compounds present in diluted corn stover and diluted corn starch pyrolysis liquors. Extracellular *Ph. chrysosporium* ligninolytic enzymes were responsible for this detoxification. The complete detoxification process needs longer incubation with fungal culture, since the transformations by *Ph. chrysosporium* are slow (2, 29). However, the concentrated enzymes can do in hours what fungi cultures do in weeks. Therefore, industrial fermentor procedures are needed for the large-scale production of these enzymes.

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Ligninolytic Enzymes Production by *Phanerochaete chrysosporium* in PCS Biofilm Stirred Tank Bioreactor

A paper to be submitted to Applied and Environmental Microbiology

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Abstract

Phanerochaete chrysosporium (ATCC 24725) produced lignin peroxidase (LiP) and manganese peroxidase (MnP) in defined medium with PCS biofilm stirred tank reactors. Laccase was not detected. The formation of the *Ph. chrysosporium* biofilm on plastic composite supports (PCS) was essential for the production of MnP and LiP. The bioreactor was operated as a repeat batch, and no reinoculation was required between batches. Peroxidases production was influenced by five min purging of the bioreactor with pure oxygen or continuous aerating with mixture of air and oxygen with flow rate of 0.005 L/min. Addition of veratryl alcohol and MnSO₄ on day zero with 300 rpm agitation and continuous aeration at 0.005 vvm produce the MnP on day three. Fourteen repeated batches fermentation were performed without contamination due to low pH (4.5) and aseptic techniques employed.

Introduction

The white-rot wood-decaying basidiomycetes are capable to produce lignin-degrading enzymes laccase, lignin peroxidase, and manganese peroxidase, or at least one of these enzymes (17, 48). These enzymes have been intensively studied in the *Phanerochaete chrysosporium* (6). The lignolytic system in *Ph. chrysosporium* is complex. It includes

numerous enzymes or associated activities such as peroxidases, H_2O_2 generating oxidases (e.g, glucose oxidase and methanol oxidase), laccase, cellobiose dehydrogenase, glyoxal oxidase, aryl alcohol oxidase, *trans*-membrane methyl transferase, *trans*-membrane redox potential and various low molecular weight compounds (6, 22). The lignin peroxidase (LiP) and manganase peroxidase (MnP) are families of isoenzymes as extracellular proteins (5). The ratio between the isoenzymes changes with the culture age and culture conditions (21, 38, 50).

Oxygen, temperatures, nutrients and inducer compounds and other factors can affect the peroxidase production in *Ph. chrysosporium* (18, 23). Veratryl alcohol (3,4-dimethoxybenzyl alcohol; VA) is synthesized *de novo* from glucose early on the secondary phase of *Ph. chrysosporium* cultures (22, 11), but the addition of the VA to cultures broth of white-rot fungi leads to increase of LiP (8,18, 39). Addition of manganese (Mn^{2+}) to the white-rot fungi cultures was found to induce the production of MnP to higher levels than the non-induced cultures (20, 53). The peroxidases were also found to be increased by dimethoxybenzyl-amine which was cleavage to veratryl alcohol and then to veratryl aldehyde (VAD) (33). Temperature and nutrients were also majors' factor, which appears to promote the production of the peroxidase (23). In *Ph. chrysosporium* shake flask culture, ligninase production correlates to limitation of nitrogen or carbohydrate and ligninase is suppressed by excess nutrients (18). Purging with pure oxygen markedly enhances the production of H_2O_2 in white-rot cultures (19), so supplying the oxygen to *Ph. chrysosporium* cultures instead of air was found to increase the lignin degradation and LiP activity (18). However, the continuous supplying with oxygen promotes LiP and MnP production and decay in submerged cultures of *Ph. chrysosporium* (14).

The previous culture conditions and several culture methods have been employed to scale up peroxidases production. The shallow stationary cultures, agitated cultures and cell immobilization bioreactors have been evaluated (2, 10, 27, 30, 33, 47, 52, 53). Under both stationary and agitated incubation the activity of ligninase reaches a maximum on days 5 to 6 but, the attempts to scale up production result in lower activity (47). The immobilization of *Ph. chrysosporium* mycelia was more effective in promoting cell growth and lignin peroxidase production improved up to 8.1 U/ml compared to 0.075 U/ml in conventional stationary liquid culture (2, 44, 47).

Biofilms are the natural form of cell immobilization (7). The types of reactors that rely on immobilized cell or biofilm include trickling filters, fixed-film or fixed-bed bioreactors, fluidized-bed reactors, and rotating bioreactors (10, 14, 24, 37, 44). However, work is still being carried out on the development of an optimal bioreactor configuration and conditions to increase *Ph. chrysosporium* ligninolytic enzyme production. Several studies have demonstrated the effectiveness of the plastic composite support (PCS) biofilm reactor to increased end-product production rate, minimal lag phase, tolerance to high concentration of nutrient, reduced requirement of micronutrients, and increased cell density for ethanol, lactic acid, and succinic acid production (9, 12, 13, 26, 49, 51).

In this study the bioreactor design of Cotton et al. (9) was employed. The PCS tubes were fixed to the agitator shaft in bioreactor to develop *Ph. chrysosporium* PCS biofilm. *Ph. chrysosporium* attached and covered the whole PCS tubes. Biofilm thickness was controlled by agitation speed. The PCS biofilm increases the cell interfacial contact with culture fluid, decreases the shear force, and permits medium to circulate through the PCS tubes. Also, in this study, the effect of agitation, oxygen flow and addition of veratryl aldehyde, enzyme

inducers such as veratryl alcohol, manganese (Mn^{2+}), and their combination to increase the ligninolytic enzymes of *Ph. chrysosporium* in PCS stirred tank biofilm reactor were investigated.

Material and methods

Microorganism and inoculum preparation

Phanerochaete chrysosporium (ATCC 24725) was obtained from American Type Culture Collection (Manassas, VA). The *Ph. chrysosporium* was maintained on potato dextrose agar slants at 4°C. The active *Ph. chrysosporium* was cultured on media containing 10.0 g/l of malt extract, 2.0 g/l of peptone, and 2.0 g/l of yeast extract (Difco lab, Deroit, MI), 10.0 g/l of glucose, 1.0 g/l of asparagine, 2.0 g/l of KH_2PO_4 , 1.0 g/l of $MgSO_4 \cdot 7H_2O$, 0.001 g/l of thiamin and 20 g/l of agar (Sigma Chemical Co. St. Louis, MO) (47). The slants were incubated for six days at 39°C. Spores production was prepared by suspension in sterile water followed by passage through sterile glass wool to remove mycelia. Finally, the inoculum spores concentration was determined by measuring absorbance at 650 nm.

Medium preparation

Peroxidase production medium, which contained 20.0 g/l of glucose, 0.22 g/l of ammonium tartrate, 2.0 g/l of KH_2PO_4 , 5.0 g/l of $MgSO_4 \cdot 7H_2O$, 0.1 g/l of $CaCl_2 \cdot H_2O$, 1.2 g/l of acetic acid, 0.4 g/l of NaOH (43), 11.7 ml of trace elements (47), 1.0 g/l of Tween 80 and 25 ml of the spore suspension (0.83/ml at A_{650}) was used for all batch experiments when not indicated otherwise in the text. The medium was prepared in 90 L, sterilized in a B-Braun 100-D fermentor (Allentown, PA) with continuous agitation for 25 min at 121°C. The trace

elements solution was added filter sterilized. The sterilized media was aseptically transferred into two sterilized 50 L carboys equipped with a carboy filling port, a medium delivery line with a liquid break, and an air vent capped with a 0.45 μm air filter for storage.

Plastic composite supports (PCS)

PCS tubes composed of 50% (wt/wt) of polypropylene, 40% (wt/wt) of ground dried soybean hull (Cargill Soy Processing Plant, Iowa Falls, IA), 5% (wt/wt) dried bovine albumin (Proliant Corp., Ames, IA), 5% (wt/wt) yeast extract (Ardamine Z from Sensient Flavor, Juneau, WI) and mineral salts were produced according to Ho et al. (25). These dry ingredients were mixed in separate container prior to being poured into extruder hopper. The twin screw co-rotating Brabender PL2000 extruder (model CTSE-V; C. W. Brabender Instruments, Inc; South Hackensack, N. J.) was operated at a rate of 11 rpm, barrel temperatures of 200, 220, and 200°C, and a die temperature of 167°C to a continuous tube. The PCS tubes had a wall thickness of 3.5 mm and an outer diameter of 10.5 mm.

PCS biofilm stirred tank bioreactor

The bioreactor design of Cotton et al (9) was employed (Figure 1). Six PCS tubes each 10 cm lengths were stacked in three rows of two parallel tubes then bound to the agitator shaft in a grid fashion. The bioreactor was a computer controlled New Brunswick Bioflo 3000 (Edison, NJ) equipped with controllers of the pH, temperature, agitation and dissolved oxygen. The 1.2 L vessel (inside diameter of 12 cm) was equipped with filtered sterilized air inlet and outlet. The broth removal port was connected with two branched lines to withdraw a sample and to remove all culture media from the vessel. The reactor was sterilized with water

for 1.25 hr at 121°C. After sterility and cool down, the bioreactor was drained and sterilize medium was pumped to the bioreactor and kept overnight before inoculation to check sterilization.

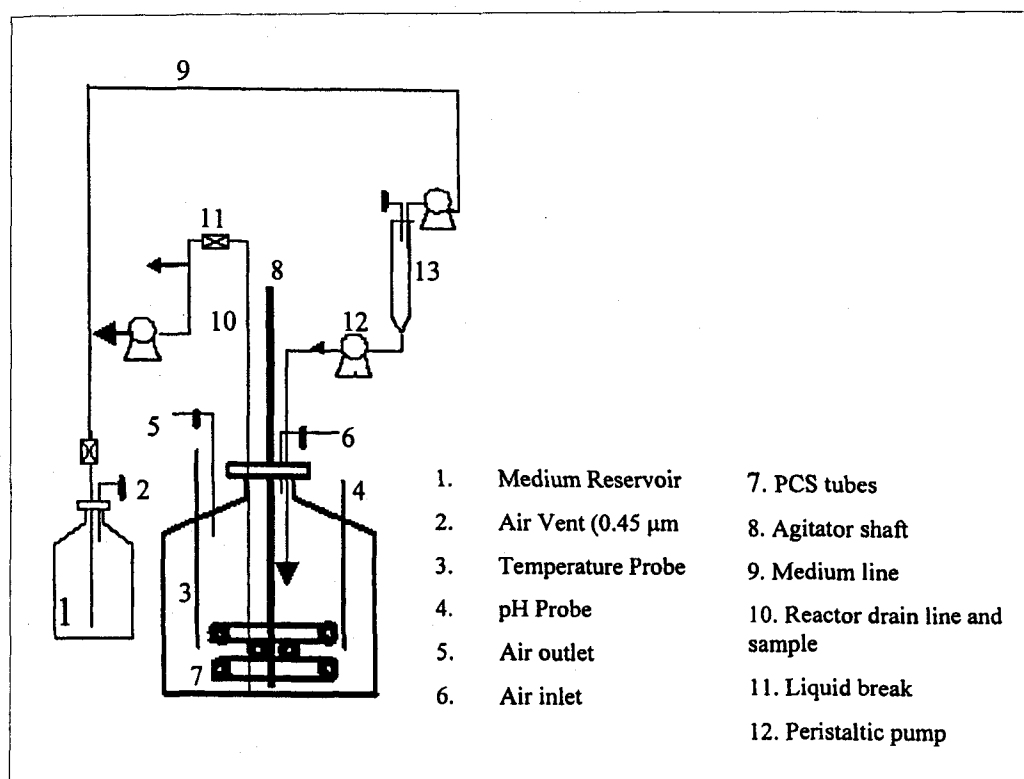


Figure 1. A schematic of the bioreactor design.

Batches Protocols

The peroxidase production was evaluated in fourteen different repeated batch fermentations at 39°C with one-liter working volume, and 6 day incubations per batch. Three samples were collected from each batch on days zero (control), three and six.

Table 1 describes the treatments evaluated. Four batch fermentations (P_1V_3 1, P_1V_3 2, P_1VA_3 3 and P_1M_3 4) investigated the effect of adding on day three 3 mM of veratryl alcohol, veratryl alcohol with limited carbon source (10 g/l of glucose instead of 20 g/l), veratryl aldehyd, and $MnSO_4$, respectively. In P_1V_3 1 the bioreactor was inoculated with 25 ml spores suspension (0.83/ml at A_{650}) (29). This was the only inoculation used for the study. After each batch, the bioreactor was drained and filled with sterile water. The agitation was increased to 500 rpm for 20 min to remove excess biofilm formed on PCS tubs, and then the bioreactor was drained again and refilled with fresh sterile media. No reinoculation was necessary, because the mycelia of the *Ph. chrysosporium* remain on and in the PCS tubs attached to the agitator shaft.

The repeated batch fermentations also investigated the effect of increasing the agitation to 300 rpm with spiking on day three 3 mM of veratryl alcohol for (P_3V_3 5), veratryl aldehyde for (P_3VA_3 6) or $MnSO_4$ for (P_3M_3 7). All other fermentations were performed as described with P_1V_3 1.

The effect of continuous oxygen addition was also investigated at 300 rpm with spiking on day three 3 mM of veratryl alcohol for (C_3V_3 8), veratryl aldehyd for (C_3VA_3 9), or $MnSO_4$ for (C_3M_3 10). Finally, the addition of enzyme inducers at time zero was evaluated with continuous oxygen addition, 300 rpm agitation, and spiking 3 mM of veratryl alcohol for (C_3V_0 11), veratryl aldehyd for (C_3VA_0 12), $MnSO_4$ for (C_3M_0 13), or mixture of veratryl alcohol and $MnSO_4$ for (C_3VM_0 14).

Table 1. Repeated batches abbreviation and fermentation conditions evaluation.

Batch abbreviation	Agitation speed (rpm)	Time of addition						Aeration conditions
		On day3			On day 0			
		V ^a	VA ^b	M ^c	V	VA	M	
P ₁ V ₃ 1	120	+ ^e	- ^f	-	-	-	-	Purging ^d
P ₁ V ₃ 2 ^g	120	+	-	-	-	-	-	Purging
P ₁ VA ₃ 3	120	-	+	-	-	-	-	Purging
P ₁ M ₃ 4	120	-	-	+	-	-	-	Purging
P ₃ V ₃ 5	300	+	-	-	-	-	-	Purging
P ₃ VA ₃ 6	300	-	+	-	-	-	-	Purging
P ₃ M ₃ 7	300	-	-	+	-	-	-	Purging
C ₃ V ₃ 8	300	+	-	-	-	-	-	Continuous ^h
C ₃ VA ₃ 9	300	-	+	-	-	-	-	Continuous
C ₃ M ₃ 10	300	-	-	+	-	-	-	Continuous
C ₃ V ₀ 11	300	-	-	-	+	-	-	Continuous
C ₃ VA ₀ 12	300	-	-	-	-	+	-	Continuous
C ₃ M ₀ 13	300	-	-	-	-	-	+	Continuous
C ₃ VM ₀ 14	300	-	-	-	+	-	+	Continuous

^a Three mM of veratryl alcohol in culture broth^b Three mM of veratryl aldehyd in culture broth^c Three mM of MnSO₄ in culture broth^d Bioreactor was purged for five minutes with pure oxygen at 0.005 vvm on day zero then daily on day three^e Addition of inducer^f No addition of inducer^g This batch only received limited carbon source (glucose 10g/l)^h Continuous aeration with mixture of air and oxygen at 0.005 vvm

Samples analysis

To determine enzyme activity, each batch was aseptically sampled (5 ml) on days 0, 3, and 6. Each sample was centrifuged at 3000 x g for 10 min at 4°C and the supernatant was evaluated for extracellular protein and enzyme activity. Oxygen consumption and change in pH were monitored via probes in bioreactor connected to computer.

Lignin peroxidase (LiP)

The level of LiP activity was determined using veratryl alcohol as substrate (47). The assay mixture contained 2 mM veratryl alcohol and 0.4 mM H₂O₂ in 50 mM sodium tartrate buffer (pH 2.5), and 200 µl culture supernatant. Oxidation of veratryl alcohol was measured as the increase in absorbance at 310 nm ($\epsilon_{310}=9,300 \text{ M}^{-1} \text{ cm}^{-1}$). The increase in $A_{310 \text{ nm}}$ was monitored during 300 sec at 37°C using a DU 640 UV/Vis Beckman spectrophotometer equipped with kinetics Soft-Pac No. 517033 for measuring enzyme activity (Beckman Instruments, Inc. Fullerton, CA). One unit (U) of LiP activity was defined as 1 µmol of veratryl alcohol oxidized in 1 min under defined conditions, and activities were reported as U/ml.

Manganese peroxidase (MnP)

The level of MnP activity was determined by monitoring the enzyme oxidation of Mn²⁺ to Mn³⁺ (40). The assay mixture contained 200 µl culture supernatant, 0.1 M sodium tartrate pH (5.0), 0.1 mM H₂O₂, and 0.1 mM MnSO₄. The product, Mn³⁺, forms a transiently stable complex with tartaric acid, showing a characteristic absorbance at 238 nm ($\epsilon=6500$). Reactions were initiated by addition of H₂O₂. The increase in $A_{238 \text{ nm}}$ was monitored during

300 sec at 37°C using a DU 640 UV/Vis Backman spectrophotometer equipped with kinetics Soft-Pac No. 517033 for measuring enzyme activity. One unit (U) of MnP activity was defined as 1 μmol of the Mn^{2+} oxidized in 1 min under defined conditions, and activities were reported as U/ml.

Laccase activity

The level of laccase was determined using 2,2'-azino-*bis*(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) as substrate (32). The reaction mixture contained 200 μl culture supernatant and 2 mM in 0.1 M sodium citrate buffer (pH 3.0). Oxidation of ABTS was monitored as the absorbance increase at 420 nm ($\epsilon=36,000 \text{ M}^{-1} \text{ cm}^{-1}$) by using a DU 640 UV/Vis Backman spectrophotometer equipped with kinetics Soft-Pac No. 517033 for measuring enzyme activity. One unit (U) of laccase activity was defined as 1 μmol of ABTS oxidize in 1 min under defined conditions, and activities were reported as Uml^{-1} .

Extracellular Protein determination

The concentrations of extracellular protein were assayed according to Lowery reagent modified method (34) after 0, 3 and 6 days for each batch. The reaction mixture was determined spectrophotometer at 750 nm by using a Spectrasonic 20 (Milton Roy, Rochester, NY). Bovine serum albumin was used as a standard for extracellular protein (50 to 400 $\mu\text{g/ml}$; $r = 0.999$).

Statistical analysis

Analysis for the enzyme activity was performed by using analysis of variance for a

completely randomized design using JMP package. Comparison of pair of mean was done using the Tukey-Kramer LSD method with 5% level of significance. Determinations were made in replicates of three fermentation batches.

Results

The concentrations of extracellular protein, laccase, MnP and LiP produced by *Ph. chrysosporium* PCS biofilm fermentation in defined medium were determined among the 14 batches; however, no laccase was detected. The enzymes production and extracellular protein were determined during the fermentation days 0, 3 and 6 in each batch and then the enzyme production and extracellular protein produced among all batches were compared for maximum production.

Analysis of variance for the 14 repeated batches of extracellular protein was statistically significant $F(13, 41) = 51.2412, p < 0.0001$ (Figure 2). Tukey-Kramer pairwise comparison tests were conducted to define the significant pairwise batch. The mean concentrations of extracellular protein released were reported descending (Figure 2) for the 6 day of fermentation with each batch. The mean concentration demonstrated that C_3V_0 11, C_3VA_3 9, C_3V_3 8 and P_1V_3 1 with means of 0.03 0.02967, 0.0276, and 0.0270 mg/ml, respectively, were not significantly different (LSD of 0.0059 mg/ml). Therefore, the extracellular protein production within the batches maybe grouped as C_3V_0 11, C_3VA_3 9, C_3V_3 8, P_1V_3 1 > P_3M_3 7, P_3V_3 5, C_3M_0 13, P_1VA_3 3, P_1V_3 2, P_1M_3 4 > P_3VA_3 6, C_3VM_0 14, C_3M_3 10, C_3VA_0 12 according to the mean values of extracellular protein production.

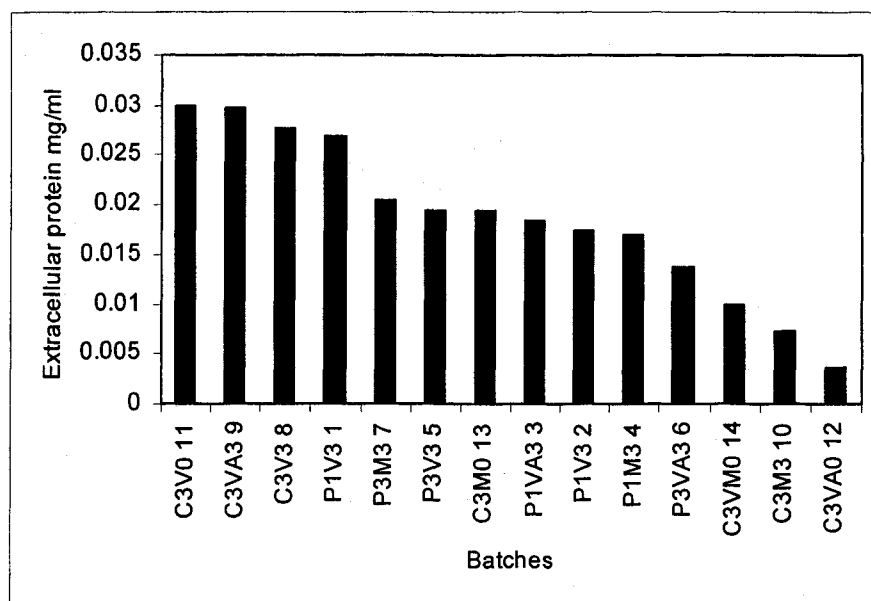


Figure 2. The concentrations of extracellular protein produced on day 6 for repeated batch fermentations arranged descending order. Each value is the average of replicates.

In MnP production among the 14 repeated batches analysis of variance was statistically significant $F(13, 41) = 9.8134, p < 0.0001$ (Figure 3). Tukey-Kramer pairwise comparison test were conducted to define the significant pairwise batch fermentation. $P_3VA_3 6$ demonstrated the highest MnP production on day 6 with mean 0.0567 U/ml. The MnP production with $C_3VM_0 14$, $C_3VA_3 9$, $P_1V_3 2$ and $C_3VA_0 12$ were 0.02533, 0.0187, 0.0045 and 0.0010 U/ml, respectively, which were significantly different from the MnP produced with $P_3VA_3 6$ (LSD of 0.0295 U/ml). Whereas, the MnP production for $C_3M_3 10$, $C_3V_3 8$, and $C_3M_0 13$, which were almost equivalent with mean 0.0497, 0.0493, and 0.0490 U/ml, respectively, were significantly different from $C_3VA_3 9$, $P_1V_3 2$, and $C_3VA_0 12$.

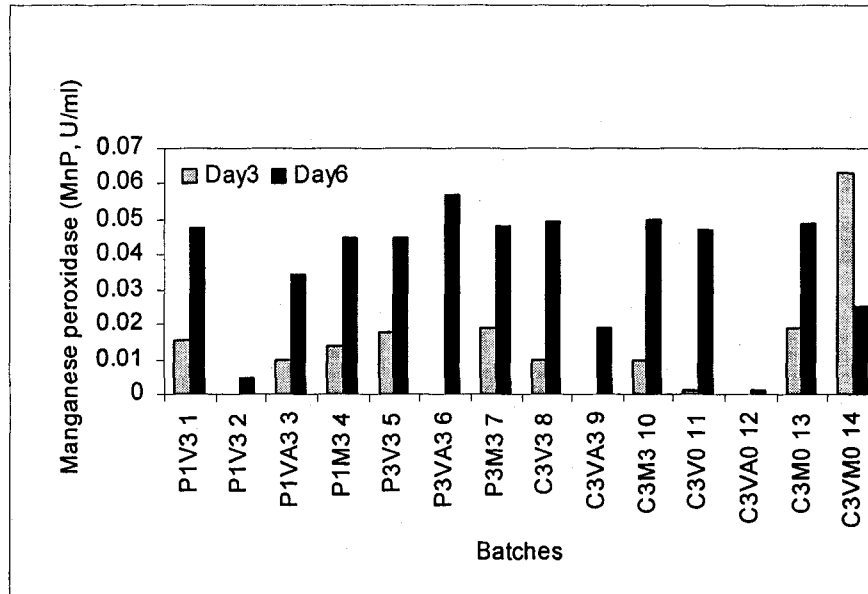


Figure 3. The production average of MnP among the 14 repeated batches fermentations on day 3 and 6. Each value is the average of replicates.

The P_3M_3 7, P_1V_3 1, C_3V_0 11, P_3V_3 5, P_1M_3 4, and P_1VA_3 3 with means 0.0480, 0.0477, 0.0473, 0.0450, 0.0447, and 0.0347 U/ml, respectively, were significantly different from P_1V_3 2 and C_3VA_0 12. Therefore, the MnP production within the batches maybe grouped as P_3VA_3 6 > C_3M_3 10, C_3V_3 8, C_3M_0 13 > P_3M_3 7, P_1V_3 1, C_3V_0 11, P_3V_3 5, P_1M_3 4, P_1VA_3 3, C_3VM_0 14, C_3VA_3 9, P_1V_3 2, and C_3VA_0 12 according to the production of the MnP.

Analysis of variance for the 14 batches of LiP production was statistically significant $F(12, 38) = 137699, p < 0.0001$ (Figure 4). Tukey-Kramer pairwise comparison tests were conducted to define the significant pairwise batch. The C_3V_0 11 and C_3V_3 8 demonstrated the highest LiP production on day 6 with means 0.0500 and 0.0473 U/ml. The productions with respect to P_3VA_3 6, C_3M_3 10, C_3M_0 13, C_3VA_0 12, and C_3VM_0 14 with means 0.0263, 0.0193, 0.0157, 0.0150, and 0.0013 U/ml, respectively, were significantly different from C_3V_0 11 and C_3V_3 8 (LSD of 0.0206 U/ml of LiP). The P_3V_3 5, P_1V_3 2 and P_1V_3 1 with

means of 0.0453, 0.0437, and 0.0417 U/ml, respectively, were significantly different from C_3M_3 10, C_3M_0 13, C_3VA_0 12, and C_3VM_0 14, whereas P_1M_3 4 (mean 0.0363 U/ml) is significantly different from C_3M_0 13, C_3VA_0 12, and C_3VM_0 14. Finally, P_1VA_3 3, C_3VA_3 9 and P_3VA_3 6 with means 0.0303, 0.0300, and 0.0263 U/ml, respectively, are significantly different only from C_3VM_0 14. Thus, according to the decrease in the LiP production, the batches maybe grouped as C_3V_0 11, C_3V_3 8 > P_3V_3 5, P_1V_3 2, P_1V_3 1 > P_1M_3 4 > P_1VA_3 3, C_3VA_3 9, P_3VA_3 6, C_3M_3 10, C_3M_0 13, C_3VA_0 12, and C_3VM_0 14.

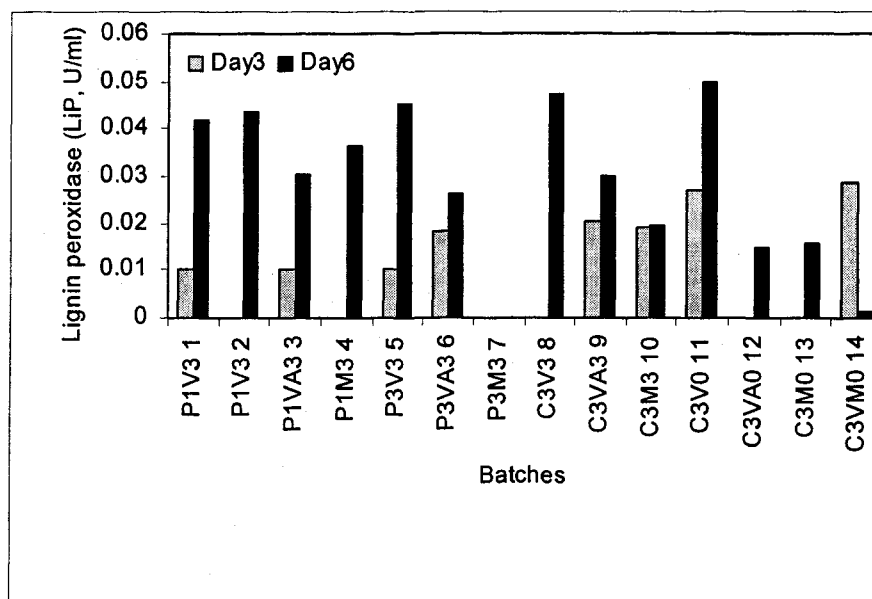


Figure 4. The production average of LiP among the 14 repeated batches fermentations on day 3 and 6. Each value is the average of replicates.

Also, comparison of the mean MnP production between days 3 and 6 demonstrates that the mean MnP production from all batches were higher in day 6 except for C_3VM_0 14 (Figure 3). The LiP production results were also higher on day 6 with all batches except with

C₃VM₀ 14, which was higher on day 3 rather than day 6 (Figure 4). The differences in the mean production between days 6 and 3 for repeated batch fermentations for MnP and LiP are shown in figure 5. The average of MnP production for C₃VM₀ 14 was 0.06323 U/ml on day 3 and dropped down on day 6 to 0.02533 U/ml. Similarly, the LiP production was higher on day three with 0.0287 U/ml and decreased to 0.004 U/ml on day 6 (Figure 5).

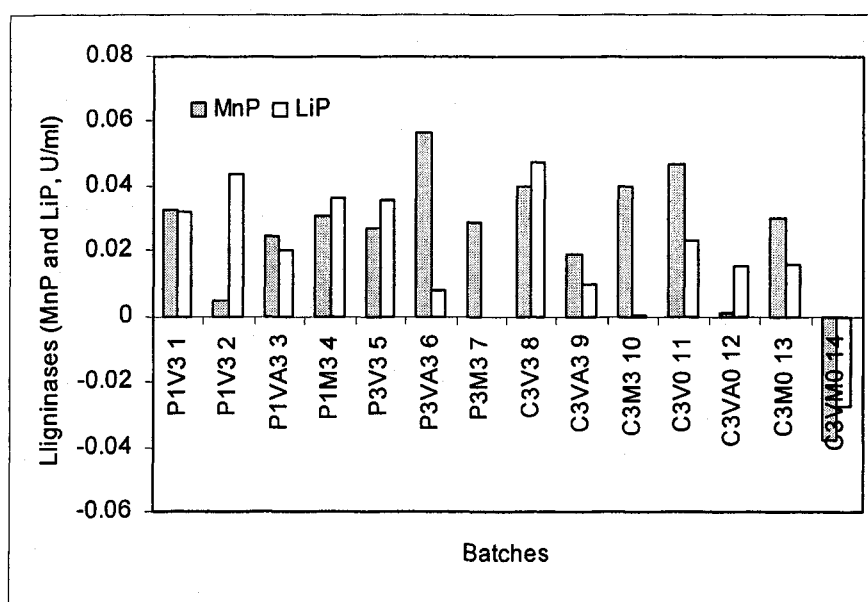


Figure 5. The difference in enzyme production mean between day 6 and 3 for each repeated batch fermentation for MnP and LiP. Each value is the average of replicates

Discussion

To our knowledge, this is the first report aimed to employ wild type *Ph. chrysosporium* with stirred tank bioreactor to produce ligninolytic enzyme. The results demonstrate that *Ph. chrysosporium* is able to develop biofilm and produce ligninase in PCS biofilm stirred tank reactors. PSC bioreactors have been shown to stimulate the microbial

attachment (12), and help prevent wash-out of biomass from the reactor (41). The PCS have also been shown to stimulate the biofilm formation and enhance the productivity of the end products (9, 12, 13, 51). In this study, the PCS stimulate the formation of *Ph. chrysosporium* biofilm and the production of ligninase in 14 repeated batches. In each batch, the PCS biofilm become significantly noticeable on day 2 and thick on day 6. The developed biofilm was important in ligninase production hence, for most batches, the production of the enzyme started on day three after the biofilm formation.

The production of both LiP and MnP enzymes depend on nutrient limitation (16, 21, 45), and different conditions between the batches (Figure 6). For example, on day three of the enzymes production, there is no relationship (pattern) between the production of LiP and MnP among the batches as shown in figure (6a). However, negative relationship might be concluded among specific batches. For example, some batches (C_3V_3 8, C_3M_0 13, P_1M_3 4 and P_3M_3 7) revealed low productions for MnP and zero production for LiP. The opposite was observed for P_3VA_3 6 and C_3VA_3 9 with no production for MnP and low production of LiP.

Whereas day 6 results clearly demonstrated 3 mM veratryl alcohol alone was sufficient to induce both MnP and LiP (Figure 6b). Furthermore, 3 mM $MnSO_4$ consistency induced MnP with little or no LiP production. Continuous vs. purged oxygen addition demonstrated no correlation to enzyme production with both procedures demonstrating excellent enzyme production (C_3V_0 11, P_1V_3 1 and C_3VA_3 9). Finally, no effect on enzyme production was observed for increasing agitation rates (120 vs. 300 rpm). Veratryl aldehydes induction of LiP and MnP was affected by oxygen addition, with continuous aeration significantly reduced enzyme production (C_3VA_0 12 and C_3VA_3 9) compared to purged

aeration (P₁VA₃ 3 and P₃VA₃ 6). This may be due to the oxidation of veratryl aldehydes to veratryl acid in the presence of continuous oxygen addition.

The *Ph. chrysosporium* PCS biofilm produced extracellular proteins with MnP and LiP enzymes in all batches. The *Ph. chrysosporium* produces very low level of laccase when it grew on glucose (6). However, laccase activity was demonstrated slightly higher levels in the concentrated culture fluids when the *Ph. chrysosporium* was grown on cellulose (46). The laccase determined in plate assay as well as a spectrophotometer, which was recorded 0.0031 U/ml (46). However, no laccase activity had been detected in our work.

The ligninolytic system in white-rot fungi, *Ph. chrysosporium*, has been considered noninducible by lignin and the growth in the presence of low concentration of a synthetic lignin dose not significantly increase the rate of CO₂ evolution from lignin (28). Also, a limited nutrients condition for fungus production of ligninase is well documented (23). In this study, all batches were performed under defined limited nitrogen media and different inducers, agitation and oxygen addition, which caused the variations the enzyme production. The addition of VA to the culture of various white-rot fungi has repeatedly been found to increase LiP enzyme titers, which is explained as protecting LiP from H₂O₂-dependent reactions by offering a good reducing substrate (15, 31). The MnSO₄ in white-rot fungi culture is considered as a regulatory affecter for the production of LiP and MnP (22, 40). Also, it has been reported that the manganese inhibited the endogenous production of veratryl alcohol (VA) in the white-rot (22). This observation might account for the increment the MnP production and reduction in the LiP production in P₁M₃ 4, P₁VA₃ 3, C₃M₃ 10, and C₃M₀ 13. In other word, the addition of mangenase (Mn⁺²) increases the MnP production (Figure 6), but inhibited the VA production, which protect the LiP from the H₂O₂-dependent

reactions. Thus, in the absence of VA, the H_2O_2 -dependent reactions degrade the LiP, while the MnP production continues.

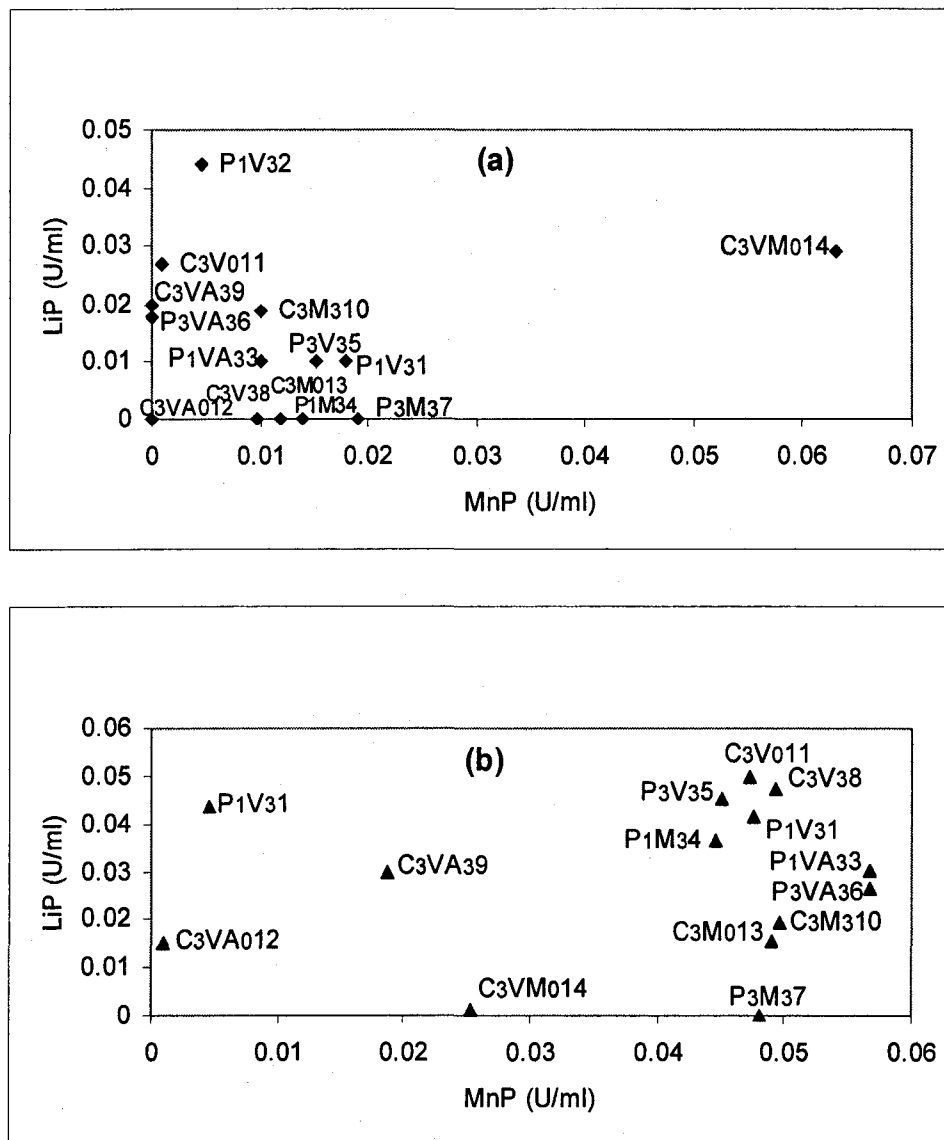


Figure 6. The pattern between the LiP and MnP production within the fourteen repeated batches fermentation (a) on day 3 and (b) on day six.

Various roles were suggested for veratryl alcohol (VA) in *Ph. chrysosporium* culture. Some studies considered VA as the real inducer of lignin peroxidase, which is needed to oxidize VA to veratryl aldehyde (VAD). The addition of VAD instead of VA to *Ph. chrysosporium* culture delays enzyme production as indicated in P₁VA₃ 3, P₃VA₃ 6, C₃VA₃ 9 and C₃VA₀ 12. Such results support the claim that VA will be formed *de novo* to induce and protect lignin peroxidase from the H₂O₂-dependent reactions (11, 31).

The biofilm thickness, aeration, and shear stress have been shown to be the key parameters for controlling lignin peroxidase production. The biofilm thickness of *Ph. chrysosporium* was identified as the main scale up parameter in fixed trickle bed reactor that produced 0.11 U/ml LiP activity after 200 hr (4). Moreover, when the *Ph. chrysosporium* I-1512 (hypersecretory strain) was immobilized in an airlift bioreactor and supplying with continuous aeration (mixture of air and oxygen) with flow rate of 5 L/h the MnP production reached 6.6 U/ml (24). Although the mechanism for the effect of oxygen is not clear, it is possible that the oxygen positively affects MnP induction of *mnp* gene transcription (20). Earlier reports showed that supplying *Ph. chrysosporium* culture with pure oxygen and agitation enhanced lignin degradation three fold (1, 30).

Agitated conditions have been thought to cause different effects on the ligninolytic activity in *Ph. chrysosporium* culture flasks and bioreactor (3, 35, 38, 42). In a pneumatically agitated bioreactor, high activity of MnP and LiP were obtained 1.812 and 4.500 U/ml, respectively (3). However, negative effect of agitation on enzyme production (0.175 U/ml) was found in stirred tank reactor, which might result due to the shear stress effect (35). The effect of the low (120 rpm) and high (300 rpm) agitation with purging or continuous aeration on the *Ph. chrysosporium* PCS biofilm culture showed no significant difference in MnP and

LiP enzymes production. The P₁V₃ 1, P₃V₃ 5 and C₃V₀ 11 demonstrated MnP activities of 0.045, 0.047 and 0.047 U/ml, respectively, and 0.019, 0.027 and 0.03 U/ml LiP activities, respectively. *Ph. chrysosporium* ligninases productions in stationary or agitated culture with limited nitrogen medium were enhanced to reach maximum on day six (31, 36, 47). However, 300 rpm agitation, addition of VA and manganase (Mn⁺²) on day zero and continuous aeration conditions (C₃VM₀ 14) stimulated the production of MnP and LiP on day 3, which reached 0.063 and 0.025 U/ml and decreased on day 6 to reached 0.025 and 0.001, respectively (Figure 3,4).

In conclusion, all different batch conditions in this study enhanced *Ph. chrysosporium* MnP and LiP enzymes synthesis. Through the present study, C₃V₀ 11 and C₃V₃ 8 considered as good choices for the production of both enzymes MnP activity of 0.0473 and 0.0493 U/ml and Lip activity of 0.03 and 0.0277 U/ml, respectively. Further, the conditions of C₃VM₀ 14 (developing PCS biofilm, continuous aeration, 300 agitation, and adding 3 mM of VA and manganase (Mn⁺²) to the medium on day zero) were shown to be effective for the early production of MnP. These culture conditions are thought to be effective for the scale up of this culturing technique. Decreasing the fermentation time can result in significant cost saving to the industry; therefore, the conditions of C₃VM₀ 14 (Figure 6) could be considered the best choice to reduce fermentation time and to enhance MnP production on the day 3. Moreover, the result of C₃VM₀ 14 suggests that these conditions will be effective for scale-up production by continuous culturing technique. Thus, the decline of LiP activity observed after day six in *Ph. chrysosporium* culture was found to be correlated with the appearance of idiophasic extracellular protease activity, but the daily addition of glucose started on day six resulted in low protease levels causing stable LiP activity (15).

Acknowledgments

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General Conclusions

This study focuses on employing the biological methods to detoxify the corn stover and corn starch pyrolysis liquors, which were produced in Brown labs at Iowa State University at 400 to 500°C. The GC-MS analysis of corn stover (Dcs) and corn starch (Dst) pyrolysis liquors illustrated the presence of numerous of toxic compounds, which inhibited the microbial growth. The phenolic compounds were dominated in the Dcs due to the lignin content, while furan and fufurly derivatives were dominate in the Dst. Levoglucosan which was the target to free from the toxic compound reported approximately 0.4 and 1.0 M in Dcs and Dst, respectively. In the first paper, *Pseudomonas putida* and *Streptomyces setonii* were employed to detoxify different concentrations of Dcs and Dst as suspended cell and PCS biofilms. However, mixture of chemical compounds in Dcs and Dst limited the detoxification ability of *P. putida* and *S. setonii* suspended cells. The PCS, which produced at Dr. Pometto's lab at Iowa State University stimulated the biofilm formation for both *P. putida* and *S. setonii*. The bioreactor developing PCS biofilms enhanced the detoxification of different concentrations of 10, 25 and 50% (vol/vol) Dcs and Dst. The 10 and 25% (vol/vol) were detoxified by *P. putida* and *S. setonii* biofilms, but the 50% (vol/vol) Dcs and Dst was not detoxify. Whereas, the PCS biofilm mixed culture was effective in detoxifying the 50% (vol/vol) of Dcs and Dst. The degradation of toxic compounds was determined via different parameters including DO₂, pH consuming, total phenolic compounds reduction, UV spectra and GC-MS analysis. The bioassay was considered as a good parameter to determine the detoxification of Dcs and Dst.

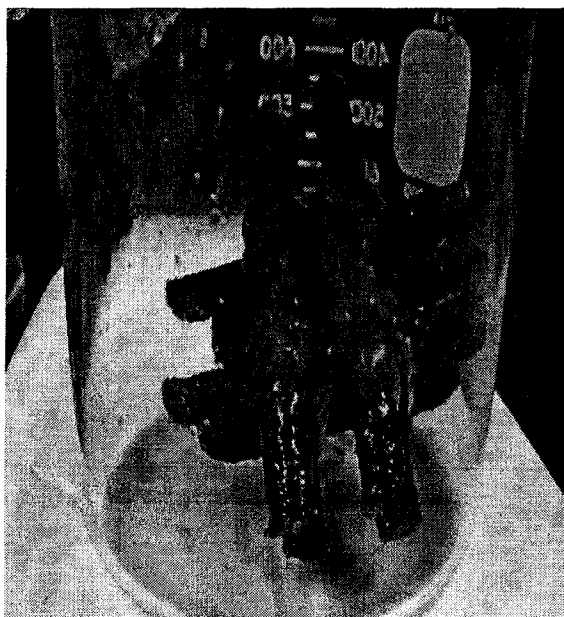
In the second paper, white-rot fungi *Ph. chrysosporium* were employed to detoxified different concentration of Dcs and Dst in flask culture under limited nitrogen medium during 14 days. *Ph. chrysosporium* was able to grow and remove the toxic compound from 10 and 25% (vol/vol), but not from 50% (vol/vol) Dcs and Dst. Ligninase and manganase peroxidases activity were detected in the culture supernatant but, no laccase activity was detected. The Dcs and Dst treated samples were analyzed by GC-MS and the results illustrated reduction in some toxic compounds pecks for 10 and 25% (vol/vol) Dcs and Dst while, in 50% (vol/vol), some pecks still appear. The total phenolic assay illustrated reduction in all Dcs concentrations. The bioassay also confirmed the detoxification of 10 and 25, but not 50% (vol/vol) Dcs and Dst. *Ph. chrysosporium* has different mechanism to oxidize toxic compounds. To confirm participation of ligninases in the detoxification, two inhibitor compounds were added to the *Ph. chrysosporium* culture. Sodium azide inhibits the oxidation of veratryl alcohol, which mediated the oxidation of toxic compounds. Whereas, cycloheximide blocks the incorporation of leucine into *Ph. chrysosporium* protein synthesis resulted in no extracellular ligninase activity. The experiment confirmed the participation of ligninases in Dcs and Dst detoxification. Concentrated ligninases remove the toxicity from 10% (vol/vol) Dcs and Dst and it was confirmed by the bioassay.

In the third paper, scale up the production of ligninases of *Ph. chrysosporium* was investigated in PCS biofilm stirred tank bioreactor. Numerous reports showed the effect of different factors on the ligninases production, such as inducers, agitation, and aeration. The study was designed as repeated batches fermentations in which each batch evaluated different factors with limited nitrogen media. The peroxidases activity under both stationary and shaken incubation reaches a maximum on day six. Thus, fourteen repeated batches were

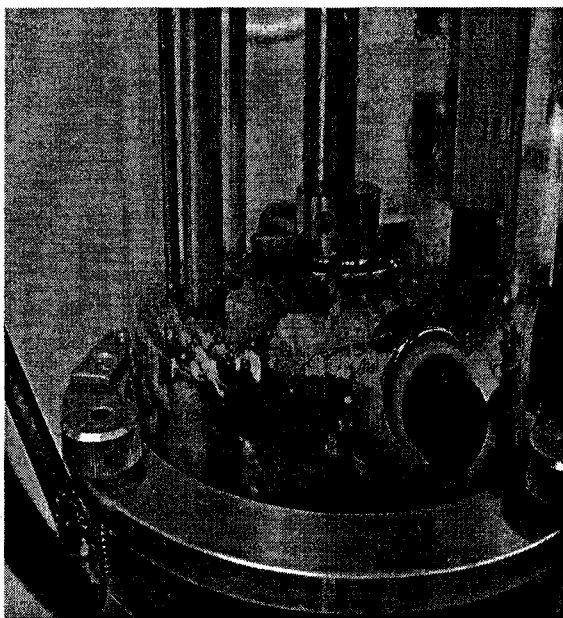
performed without contamination for each batch; the peroxidases were evaluated on day zero, three and six. The biofilm was developed on PCS tubes fixed to the agitator shaft. The ligninases activities were presence in each batch. Through the study, C_3V_0 11 and C_3V_3 8 might considered as good choices for the production of both enzymes on day six with MnP activity of 0.0473 and 0.0493 U/ml and Lip activity of 0.03 and 0.0277 U/ml, respectively. Batch fourteen, (C_3VM_0 14), in which 3 mM of two inducers, veratryl alcohol and Mn (II), were added on day zero, with 300 rpm agitation and continuous aeration, would considered as the best choice for stimulating the MnP and LiP production on day three to reach 0.063 and 0.025 U/ml, respectively. However, such activity decreased on day six to reach 0.025 and 0.001 U/ml, respectively.

Appendix

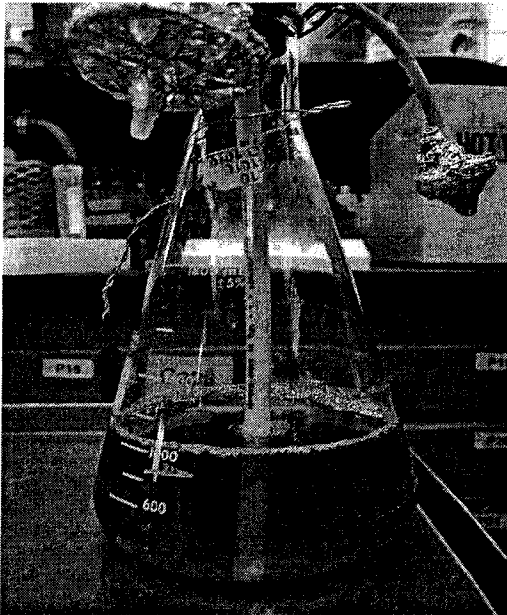
P. putida biofilm on PCS tubes fixed to the agitator shaft



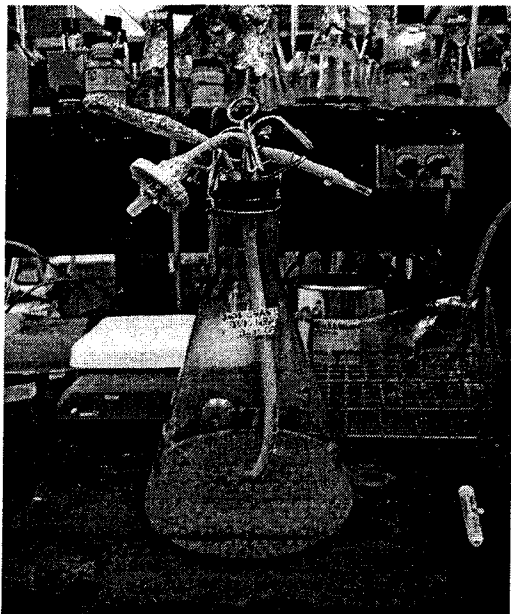
P. putida biofilm on PCS tubes after six months in cold room



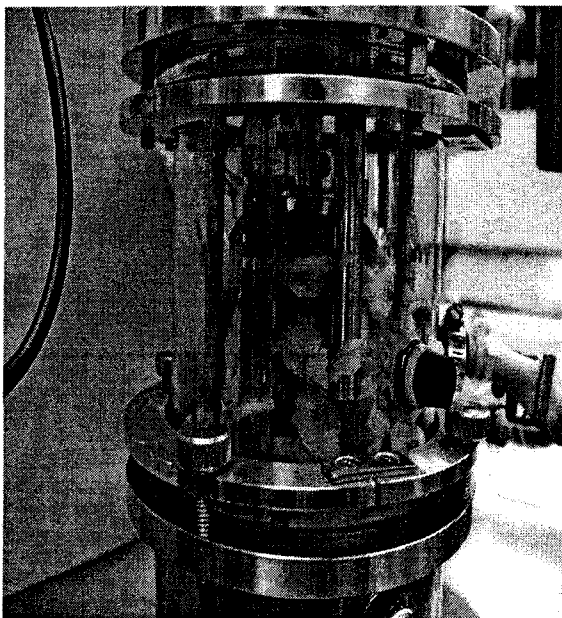
S. setonii biofilm on PCS tubes
fixed to the agitator shaft



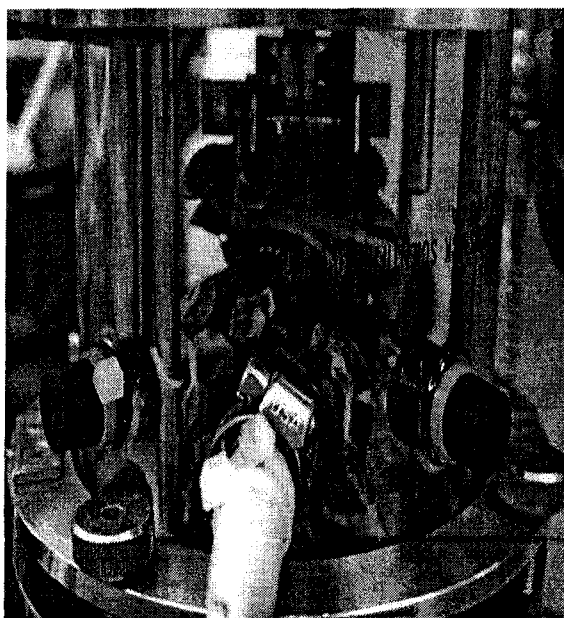
Ph. chrysosporium with 25% (vol/vol) Dcs and limited nitrogen medium on day 4



Ph. chrysosporium with 25% (vol/vol) Dcs and limited nitrogen medium on day 14



Ph. chrysosporium biofilm
on PCS tubes fixed to the
agitator shaft during
ligninases production in
stirred tank bioreactor



Ph. chrysosporium biofilm
removed from the PCS tubes
fixed to the agitator shaft to
start new batch with fresh
medium

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